GENETIC SEQUENCES HAVING METHYLTRANSFERASE ACTIVITY AND USES THEREFOR

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates generally to a genetic sequence encoding a polypeptide having methyltransferase activity and the use of the genetic sequence and/or the polypeptide to modify one or more phenotypic characteristics of a plant. More particularly, the methyltransferase of the present invention acts on flavonoids, preferably wherein the flavonoid is an anthocyanin. Even more particularly, the present invention relates to a polypeptide having *S*-adenosyl-L-methionine: anthocyanin 3' -*O*-methyltransferase or *S*-adenosyl-L-methionine: anthocyanin 3', 5'-*O*-methyltransferase activity. The present invention still further provides a genetic sequence encoding a polypeptide having methyltransferase activity derived from *Petunia, Torenia, Fuchsia* or *Plumbago* or botanically related plants. The instant invention further relates to antisense and sense molecules corresponding to all or part of the subject genetic sequence as well as genetically modified plants as well as cut flowers, parts, extracts and reproductive tissue from such plants.

DESCRIPTION OF THE PRIOR ART

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

The flower or ornamental plant industry strives to develop new and different varieties of flowers and/or plants. An effective way to create such novel varieties is through the

manipulation of flower color. Classical breeding techniques have been used with some success to produce a wide range of colors for most of the commercial varieties of flowers and/or plants available today. This approach has been limited, however, by the constraints of a particular species' gene pool and for this reason it is rare for a single species to have the full spectrum of colored varieties. For example, the development of novel colored varieties of plants or plant parts such as flowers, foliage and stems would offer a significant opportunity in both the cut flower and ornamental markets. In the flower or ornamental plant industry, the development of novel colored varieties of major flowering species such as rose, chrysanthemum, tulip, lily, carnation, gerbera, orchid, lisianthus, begonia, torenia, geranium, petunia, nierembergia, pelargonium, impatiens and cyclamen would be of great interest. A more specific example would be the development of a blue rose or gerbera for the cut flower market.

In addition, the development of novel colored varieties of plant parts such as vegetables, fruits and seeds would offer significant opportunities in agriculture. For example, novel colored seeds would be useful as proprietary tags for plants. Furthermore modifications to flavonoids common to berries including grapes and their juices including wine have the potential to impart altered style characteristics of value to such fruit and byproduct industries.

Flower color is predominantly due to three types of pigment: flavonoids, carotenoids and betalains. Of the three, the flavonoids are the most common and contribute a range of colors from yellow to red to blue. The flavonoid molecules that make the major contribution to flower color are the anthocyanins, which are glycosylated derivatives of cyanidin and its methylated derivative peonidin, delphinidin and its methylated derivatives petunidin and malvidin and pelargonidin. Anthocyanins are localised in the vacuole of the epidermal cells of petals or vacuole of sub epidermal cells of leaves.

The flavonoid pigments are secondary metabolites of the phenylpropanoid pathway. The biosynthetic pathway for the flavonoid pigments (flavonoid pathway) is well established, (Holton and Cornish, *Plant Cell 7*: 1071-1083, 1995; Mol *et al.*, *Trends Plant Sci. 3:* 212–

217, 1998; Winkel-Shirley, *Plant Physiol. 126*: 485-493, 2001a and Winkel-Shirley, *Plant Physiol. 127*: 1399-1404, 2001b) and is shown in Figures 1A and B. Three reactions and enzymes are involved in the conversion of phenylalanine to *p*-coumaroyl-CoA, one of the first key substrates in the flavonoid pathway. The enzymes are phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate: CoA ligase (4CL). The first committed step in the pathway involves the condensation of three molecules of malonyl-CoA (provided by the action of acetyl CoA carboxylase (ACC) on acetyl CoA and CO_2) with one molecule of *p*-coumaroyl-CoA. This reaction is catalysed by the enzyme chalcone synthase (CHS). The product of this reaction, 2',4,4',6', tetrahydroxy-chalcone, is normally rapidly isomerized by the enzyme chalcone flavanone isomerase (CHI) to produce naringenin. Naringenin is subsequently hydroxylated at the 3 position of the central ring by flavanone 3-hydroxylase (F3H) to produce dihydrokaempferol (DHK).

The B-ring of DHK can be hydroxylated at either the 3', or both the 3' and 5' positions, to produce dihydroquercetin (DHQ) and dihydromyricetin (DHM), respectively. The pattern of hydroxylation of the B-ring plays a key role in determining petal color, with DHK generally leading to the production of the brick red pelargonidin-based pigments, DHQ generally leading to the red/pink cyanidin-based pigments and DHM generally leading to the blue/violet delphinidin-based pigments.

The dihydroflavonols (DHK, DHQ and DHM) can also be acted upon by flavonol synthase to produce the flavonols kaempferol, quercetin and myricetin. The flavonols are colorless but act as copigments with the anthocyanins to enhance flower color.

The next step in the pathway, leading to the production of the colored anthocyanins from the dihydroflavonols, involves dihydroflavonol 4-reductase (DFR) with the production of the leucoanthocyanidins. These flavonoid molecules are unstable under normal physiological conditions and glycosylation at the 3-position, through the action of glycosyltransferases, stabilizes the anthocyanidin molecule thus allowing accumulation of the anthocyanins. In general, the glycosyltransferases transfer the sugar moieties from UDP sugars and show high specificities for the position of glycosylation and relatively low

specificities for the acceptor substrates (Seitz and Hinderer, Anthocyanins. In: *Cell Culture and Somatic Cell Genetics of Plants.* Constabel, F. and Vasil, I.K. (eds.), Academic Press, New York, USA, *5*: 49-76, 1988). Anthocyanins can occur as 3-monosides, 3-biosides and 3-triosides as well as 3, 5-diglycosides and 3, 7-diglycosides associated with the sugars glucose, galactose, rhamnose, arabinose and xylose (Strack and Wray, In: *The Flavonoids - Advances in Research since 1986.* Harborne, J.B. (ed), Chapman and Hall, London, UK, 1-22, 1993).

Glycosyltransferases involved in the stabilization of the anthocyanidin molecule include UDP glucose: flavonoid 3-glucosyltransferase (3GT), which transfers a glucose moiety from UDP glucose to the 3-*O*-position of the anthocyanidin molecule to produce anthocyanidin 3-*O*-glucoside.

In petunia and pansy (amongst others), these anthocyanins can then be glycosylated by another glycosyltransferase, UDP rhamnose: anthocyanidin 3-glucoside rhamnosyltransferase (3RT), which adds a rhamnose group to the 3-*O*-bound glucose of the anthocyanin molecule to produce the anthocyanidin 3-rutinosides, and once acylated, can be further modified by UDP: glucose anthocyanin 5 glucosyltransferase (5GT).

Many anthocyanidin glycosides exist in the form of polyacylated derivatives. Acylation may be important for uptake of anthocyanins into the vacuoles as was demonstrated by Hopp and Seitz (*Planta 170*: 74-85, 1987). The acyl groups that modify the anthocyanidin glycosides can be divided into two major classes based upon their structure. The aliphatic acyl groups include malonic acid or succinic acid and the aromatic class includes the hydroxy cinnamic acids such as *p*-coumaric acid, caffeic acid and ferulic acid and the benzoic acids such as *p*-hydroxybenzoic acid.

Acylation of the anthocyanidin 3-rutinosides with either *p*-coumaric acid or caffeic acid (Griesbach *et al.*, *Phytochemistry 30*: 1729-1731, 1991) occurs in *Petunia hybrida*. In other plant systems, acylation of flavonoids by aliphatic acids, such as malonic acid, succinic

acid and acetic acid also occur (Goto, *Tetrahedron 27:* 2413-2416, 1987; Stafford, *Flavonoid Metabolism.* CRC Press, Inc. Boca Raton, Florida, USA, 1990).

Methylation at the 3' and 3', 5' positions of the B-ring of anthocyanidin 3-(*p*-coumaroyl) rutinoside-5-glucosides occurs in petunia. It has been demonstrated in cell-free extract of flower buds of *P. hybrida* that *S*-adenosyl-L-methionine is the methyl donor and *O*-methyltransferase acts on anthocyanidin 3(*p*-coumaroyl) rutinoside-5-glucoside. Under the conditions used, no methylating activity was detected when anthocyanidins, anthocyanidin 3-glucosides, anthocyanidin 3-rutinosides, caffeic acid or *p*-coumaric acid were used as substrates (Jonsson *et al.*, *Phytochemistry 21(10)*: 2457-2460, 1982).

Methylation of the B ring of anthocyanins is controlled by the *Mt1*, *Mt2*, *Mf1* and *Mf2* loci in petunia (Jonsson *et al., Theor. Appl. Genet. 68:* 459-466, 1984b). The four enzymes thought to be encoded by each gene have been described. They catalyze both 3' and 5' *O*-methylation of the B ring. The 3'5' methylation activity is more pronounced with the *Mf1* and *Mf2* encoded enzymes (Jonsson *et al.*, 1984b, *supra*).

The *Mt* loci were thought to encode *S*-adenosyl-L-methionine:anthocyanin 3' -*O*-methyltransferase (3'FMT) and the *Mf* loci to encode *S*-adenosyl-L-methionine:anthocyanin 3', 5'-*O*-methyltransferase activity (3'5'FMT) and that the enzymes only methylate the anthocyanin 3-(p-coumaroyl) rutinoside-5-glucoside. (Jonsson *et al.*, 1982 *supra*; Jonsson *et al.*, *Planta 160:* 174-179, 1984a; Jonsson *et al.*, 1984b, *supra*). It was originally thought that the genes *Mf1* and *Mf2* could only express themselves if at least one of the genes *Mt1* or *Mt2* is represented by its dominant allele. However, biochemical studies have since contradicted these findings by showing that both enzymes were capable of methylating delphinidin 3-(p-coumaroyl)-rutinoside-5-glucosides to the corresponding malvidin pigment in *in vitro* assays (Jonsson *et al.*, *Theor. Appl. Genet. 66:* 349-355, 1983). Furthermore, the action of *Mf1* and *Mf2* was thought to be restricted to the corolla limb (Wiering, *Hort. Genen. Phaenen. 17*: 117-134, 1974).

The presence of methylated anthocyanin pigments have been reported in *Petunia sp.* (Sink (ed), *Petunia,* Springer-Verlag, Berlin, 1984; Ando *et al., Biochemical systematics and ecology, 27*: 623-650, 1999), *Plumbago sp. (inter alia,* Harborne, *Phytochemistry, 6:* 1415-1428, 1967; Harborne, *Arch Biochem Biophys, 96:* 171-178, 1962), *Vitis sp.* (Cachio *et al., American J of Ecology and Viticulture, 43*: 244-248, 1992), *Babiana stricta (*Toki *et al., Phytochemistry, 37*: 885-887, 1994), *Pinus sp.* (Andersen, *Biochemical systematics and ecology; 20*: 145-148, 1992), *Picea sp., Larix sp., Phaseolus sp.* (Hungria *et al., Plant Physiology, 97*: 751-758, 1991; Takeoka *et al., Journal of Agricultural and Food Chemistry, 45*: 3395-3400, 1997), *Solanum sp.* (Lewis *et al., J. of the Science of Food and Agriculture, 77*: 45-57, 1998), *Vaccinium sp.* (Ballington *et al., Can. J. of Plant Sci., 68*: 241-246, 1988; Skrede *et al., J of Food Science, 65*: 357-364, 2000), *Cyclamen sp.* (Webby and Boase, *Phytochemistry, 52*: 939-941, 1999), *Iris sp.* (Yabuya *et al., Euphytica, 98*: 163-167, 1997; Yabuya and Noda, *Euphytica, 103*: 325-328, 1998), *Pelargonium* sp. (Mitchell *et al., Phytochemistry, 47*: 355-361, 1998; Kobayashi *et al., Breeding Science, 48*: 169-176, 1998), *Geranium sp.* (Andersen *et al., Pytochemistry, 38*: 1513-1517, 1995), *Pisum sp.* (Crowden, *Phytochemistry, 21*: 2989-2990, 1982), *Lathyrus sp.* (Rat'kin *et al., Zh Obshch Biol, 41*: 685-699, 1980), *Clitoria sp (*Srivastava and Pande, *Planta Med, 32*: 138-140, 1977)., *Catharanthus sp.* (Carew and Krueger, *Phytochemistry, 15:* 442, 1976), *Malvia sp.* (Takeda *et al., Phytochemistry*, 28: 499-500, 1989), *Mucuna sp.* (Ishikura and Shibata, *Bot Mag (Tokyo), 86:* 1-4, 1973), *Vicia sp.* (Catalano *et al., J. Agricultural and Food Chemistry, 49*: 4568-4570, 1998; Nozzolillo *et al., Canadian Journal of Botany, 67*: 1600-1604, 1989), *Saintpaulia sp.* (Griesbach, *Phytochemistry, 48*: 829-830, 1998), *Lagerstroemia sp.* (Toki and Katsuyama, *J. Jap Soc Hortic. Sci., 63*: 853-861, 1995), *Tibouchina sp.* (Francis *et al., J Am Soc Hortic Sci, 107*: 789-791, 1982, Terahara *et al., J. Natural Products, 56*: 335-340, 1993), *Hypocalyptus sp.*(Van Wyk *et al., Biochemical systematics and ecology, 23*: 295-297, 1995), *Rhododendron sp., Linum sp., Macroptilium sp.* (Imrie and Hutton, *J. Hered., 69*: 54-56 1978), *Hibiscus sp.* (Kim *et al., Phytochemistry, 28*: 1503-1506, 1989; Kim and Fujieda, *J. Kor. Soc. Hortic. Sci., 32*: 247-255, 1991), *Hydrangea sp.* (Takeda *et al., Phytochemistry, 29*: 1089-1091, 1990), *Ipomoea sp.* (Saito *et al., Phytochemistry 41*: 1607-1611, 1996), *Cymbidium sp.* (Woltering and

Somhorst, *J. Plant Physiol., 136*: 295-299, 1990), *Millettia sp.* (Parvez and Ogbeide, *Phytochemistry, 29*: 2043-2044, 1990), *Hedysarum sp.* (Chriki and Harborne, *Phytochemistry, 22*: 2322-2323, 1983; Chriki, *Agronomie, 10*: 553-540, 1990), *Lespedeza sp., Antigonon sp.* (Tiwari and Minocha, *Vijnana Parishad Anusandhan Patrika, 23*: 305-308, 1980) and*Pisum sp.* (Crowden, *Phytochemistry, 21*: 2989-2990, 1982).

This list describes the species from which methylated anthocyanin pigments have been reported. However, it is expected that these pigments will be present in many other species.

Plant *S*-adenosyl-L-methionine-dependent *O*-methyltransferases (SAM-OMTs) are key enzymes in metabolic pathways such as phenylpropanoid and flavonoid synthesis. These enzymes facilitate the transfer of the methyl group of *S*-adenosyl-L-methionine (SAM) to the hydroxyl group of an acceptor molecule with the formation of its methyl ether derivative and *S*-adenosyl-L-homocysteine as products. The chemical mechanisms of methyl transfer reactions are identical. However, SAM-OMTs differ in their selectivity with respect to the stereochemistry of the methyl acceptor molecules, as well as the substitution pattern of their phenolic hydroxyl groups. Methylation of different substrates is generally catalysed by distinct SAM-OMTs. However, some enzymes have a broad substrate range although they will usually have a preference for a specific substrate or group of compounds.

Currently, there are over 87 plant-derived sequences encoding SAM-OMTs in the GenBank database. Practically all of these sequences contain three highly conserved consensus motifs (motifs A, B and C) exhibiting a specific spatial arrangement (Joshi and Chiang, *Plant Mol. Biol. 37*: 663-674, 1998; Ibrahim and Muzac, In *Recent advances of phytochemistry.* Evolution of metabolic pathways. Elsevier Science Ltd. *34*: 349- 385, 2000). Since these motifs are present in most plant SAM-OMTs regardless of substrate specificity, it is thought that they are essential for SAM binding.

By considering the length of the encoded protein and the spatial relationships between motifs A and B and motifs B and C, the plant SAM-OMTs can be grouped into two

distinct classes. Group I contains all the CCoAOMTs (caffeoyl-CoA SAM-OMTs) and exhibits a specific spatial arrangement of 19 amino acids between motifs A and B, and 24 amino acids between motifs B and C. Group II contains proteins with a distance of 52 residues between motifs A and B and 30 residues between B and C. Group II SAM-OMTs include COMTs (caffeic acid OMTs), F3'OMT (flavonoid 3'-OMT) (Gauthier *et al.*, *Plant Mol. Biol. 32:* 1163-1169, 1996), IOMTs (isoflavone OMTs) (He and Dixon, *Plant Mol. Biol. 36*: 43-54, 1998), 2'OMTs (isoliquiritigenin 2'-OMT) (Maxwell, *Plant J. 4(6)*: 971-981, 1993), IMT (inositol OMT) (Rammesmeyer *et al.*, *Arch. Biochem. Biophys. 322(1)*: 183-188, 1995), and F7OMT (flavonoid 7-OMT) (Christensen *et al.*, *Plant Mol. Biol. 36*: 219-227, 1998), among others. It is important to note at this point that those enzymes for which substrate analysis has been undertaken and for which function has been assigned are usually tested with a limited range of substrates. The flavonoid SAM-OMT sequences that have been isolated to date have all been implicated in defense responses with none being shown to have activity on anthocyanins and belong to the Group II SAM-OMTs.

CCoAOMT proteins, or Group I SAM-OMTs, vary in length between 231-248 amino acids and usually require divalent cations, such as Mg^{2+}, for catalytic activity. Group II SAM-OMTs are generally around 344-383 amino acids in length and do not require divalent cations. The two groups share approximately 20 - 30 % amino acid identity.

In addition to the above modifications, pH and copigmentation with other flavonoids such as flavonols and flavones can affect petal color. Flavonols and flavones can also be aromatically acylated (Brouillard and Dangles, In: *The Flavonoids - Advances in Research since 1986*. Harborne, J.B. (ed), Chapman and Hall, London, UK, 1-22, 1993).

The ability to control the activity of flavonoid methyltransferases (herein after referred to as "FMT") specifically anthocyanin methyltransferases would provide a means of manipulating petal color thereby enabling a single species to express a broader spectrum of flower colors. Such control may be by modulating the level of production of an indigenous enzyme or by introducing a non-indigenous enzyme.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is provided in Table 1. A sequence listing is provided after the claims.

In accordance with the present invention, it has been determined that malvidin-based pigments appear "bluer" than delphinidin-based pigments in the same petal background. A class of methyltransferases that act on flavonoids and in particular anthocyanins have been isolated and surprisingly found to belong to the Class I SAM-OMT group instead of the Class II SAM-OMT as may have been predicted from the literature. These are referred to herein as flavonoid methyltransferases (FMT or FMTs). Examples of these novel methyltransferases include, but are not limited to, 3' FMT and 3'5' FMT. These novel FMTs can be derived from many species, for example, *Petunia sp.*, *Torenia sp. Plumbago sp.* and *Fuchsia sp.*

The present invention provides, therefore, isolated nucleic acid molecules comprising sequences of nucleotides encoding, or complementary to sequences encoding, an FMT or a mutant, derivative, part, fragment, homolog or analog thereof.

The mutants, derivatives, parts, fragments, homologs and analogs may or may not be functional. Preferably, however, they are functional.

The isolated nucleic acid molecules encoding the FMTs of the present invention are proposed to be useful in manipulating the color of plants or plant parts such as flowers,

fruits, nuts, roots, stems, leaves and seeds. Genetic modification of plants with the nucleic acid molecules of the present invention further permit altered plants wherein extracts thereof are useful as flavoring or food additives or health products including beverage or juice products. Such beverages include but are not limited to wines, spirits, teas, coffee, milk and dairy products.

Particularly, exemplified nucleic acid molecules are from Petunia (SEQ ID NO: 1, SEQ ID NO: 4, SEQ ID NO: 6 and SEQ ID NO: 26), *Torenia* (SEQ ID NO: 11) and *Fuchsia* (SEQ ID NO: 21, SEQ ID NO: 41 and SEQ ID NO: 43). The corresponding amino acid sequences are represented by SEQ ID NO: 2, SEQ ID NO: 5 and SEQ ID NO: 7 (all *Petunia*), SEQ ID NO: 12 (*Torenia*) and SEQ ID NO: 42 and SEQ ID NO: 44 (both *Fuchsia*).

Accordingly, in a preferred embodiment, the present invention provides nucleic acid molecules comprising nucleotide sequences as defined in SEQ ID NO:1 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:11 or SEQ ID NO:21 or SEQ ID NO:26 SEQ ID NO:41 or SEQ ID NO:43 or a nucleotide sequence having at least about 50% similarity thereto or capable of hybridizing to one or more of these sequences.

The nucleic acid molecules of the present invention preferably encode an amino acid sequence substantially as set forth in SEQ ID NO:2 or SEQ ID NO:5 or SEQ ID NO:7 or SEQ ID NO:12 or SEQ ID NO:22 or SEQ ID NO:42 or SEQ ID NO:44 or an amino acid sequence having at least about 50% similarity thereto.

The present invention further provides an oligonucleotide of 5-50 nucleotides having substantial similarity or complementarity to a part or region of a molecule with a nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:11 or SEQ ID NO:21 or SEQ ID NO:26 or SEQ ID NO:41 or SEQ ID NO:43 or a complementary form thereof.

A summary of sequence identifiers used throughout the specifation is provided in Table 1.

A further aspect of the present invention provides a method for producing a transgenic plant capable of synthesizing FMT, said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence which comprises a sequence of nucleotides encoding said FMT under conditions permitting the eventual expression of said nucleic acid sequence, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence. The transgenic plant may thereby produce non-indigenous FMT at elevated levels relative to the amount expressed in a comparable non-transgenic plant.

Another aspect of the present invention contemplates a method for producing a transgenic plant with reduced indigenous or existing FMT activity, said method comprising stably transforming a cell of a suitable plant with a nucleic acid molecule which comprises a sequence of nucleotides encoding or complementary to a sequence encoding an FMT activity, regenerating a transgenic plant from the cell and where necessary growing said transgenic plant under conditions sufficient to permit the expression of the nucleic acid.

Yet another aspect of the present invention contemplates a method for producing a genetically modified plant with reduced indigenous or existing FMT activity, said method comprising altering the *FMT* gene through modification of the indigenous sequences *via* homologous recombination from an appropriately altered *FMT* gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

Still another aspect of the present invention contemplates a method for producing a transgenic plant exhibiting altered inflorescence properties, said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence of the present invention, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence into an FMT.

Still a further aspect of the present invention contemplates a method for producing a plant exhibiting altered inflorescence properties, said method comprising alteration of the *FMT* gene through modification of the indigenous sequences *via* homologous recombination from an appropriately altered *FMT* gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

Even yet another aspect of the present invention extends to a method for producing a transgenic plant capable of expressing a recombinant gene encoding an FMT or part thereof or which carries a nucleic acid sequence which is substantially complementary to all or a part of a mRNA molecule optionally transcribable where required to effect regulation of an FMT, said method comprising stably transforming a cell of a suitable plant with the isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding, an FMT, where necessary under conditions permitting the eventual expression of said isolated nucleic acid molecule, and regenerating a transgenic plant from the cell.

Even still another aspect of the present invention extends to all transgenic plants or parts of transgenic plants or progeny of the transgenic plants containing all or part of the nucleic acid sequences of the present invention, or antisense forms thereof and/or any homologs or related forms thereof and, in particular, those transgenic plants which exhibit altered inflorescence properties.

Even still another aspect of the present invention extends to all transgenic plants or parts of transgenic plants or progeny of the transgenic plants containing all or part of the nucleic acid sequences of the present invention, or antisense forms thereof and/or any homologs or related forms thereof and, in particular, those transgenic plants which exhibit altered aerial parts of the plant such as sepal, bract, petiole, peduncle, ovaries, anthers or stem properties.

Another aspect of the present invention contemplates the use of the extracts from transgenic plants or plant parts transgenic plants or progeny of the transgenic plants containing all or part of the nucleic acid sequences of the present invention and, in

particular, the extracts from those transgenic plants when used as a flavoring or food additive or health product or beverage or juice or coloring.

A further aspect of the present invention is directed to recombinant forms of FMT.

Another aspect of the present invention contemplates the use of the genetic sequences described herein in the manufacture of a genetic construct capable of expressing an FMT or down-regulating an indigenous FMT enzyme in a plant.

Yet another aspect of the present invention is directed to a prokaryotic or eukaryotic organism carrying a genetic sequence encoding an FMT extrachromasomally in plasmid form.

Still another aspect of the present invention extends to a recombinant polypeptide comprising a sequence of amino acids substantially as set forth in SEQ ID NO:2 or SEQ ID NO:5 or SEQ ID NO:7 or SEQ ID NO:12 or SEQ ID NO:22 or SEQ ID NO:42 or SEQ ID NO:44 or an amino acid sequence having at least about 50% similarity to SEQ ID NO:2 or SEQ ID NO:5 or SEQ ID NO:7 or SEQ ID NO:12 or SEQ ID NO:22 or SEQ ID NO:42 or SEQ ID NO:44 or a derivative of said polypeptide.

TABLE 1

Summary of sequence identifiers

SEQUENCE ID NO:	NAME	DESCRIPTION
1	Petunia *difE* nt sequence	cDNA nucleotide
2	Petunia difE aa seq	cDNA translated sequence
3	'GAGATTT'	oligonucleotide
4	*Petunia E20* nt seq	cDNA nucleotide
5	*Petunia* E20 aa	cDNA translated sequence
6	*Petunia E33* nt	cDNA nucleotide
7	*Petunia* E33 corrected aa	cDNA amino acid (corrected)
8	1903 F	FMT specific oligonucleotide
9	1907BamHI F	FMT specific oligonucleotide
10	1907Pst R	FMT specific oligonucleotide
11	*Torenia TMT5*.nt	cDNA nucleotide
12	*Torenia* TMT5.aa	cDNA translated sequence
13	TMT5.BamHI.F	oligonucleotide
14	TMT5.PstI.R	oligonucleotide
15	OMTIf2	FMT specific oligonucleotide
16	OMTIf4	FMT specific oligonucleotide
17	OMTIr3	FMT specific oligonucleotide
18	OMTIr5	FMT specific oligonucleotide
19	dT(17)Ad2Ad1	oligonucleotide
20	GI-anchor	oligonucleotide
21	*Fuchsia FMT* nt	cDNA nucleotide
22	*Fuchsia* FMT aa	cDNA amino acid
23	OMT1f1	FMT specific oligonucleotide
24	OMT1f3	FMT specific oligonucleotide
25	OMT1r4	FMT specific oligonucleotide
26	*Petunia E33* nt (corrected)	cDNA nucleotide

SEQUENCE ID NO:	NAME	DESCRIPTION
27	Ad1	oligonucleotide
28	petD8#1	oligonucleotide
29	petD8#2	oligonucleotide
30	PMT-F	FMT specific oligonucleotide
31	PMT-R	FMT specific oligonucleotide
32	TMT-F	FMT specific oligonucleotide
33	TMT-R	FMT specific oligonucleotide
34	FucR1	FMT specific oligonucleotide
35	FucR3	FMT specific oligonucleotide
36	FucR5	FMT specific oligonucleotide
37	FucR6	FMT specific oligonucleotide
38	FucF1	FMT specific oligonucleotide
39	Tor 5' pos	FMT specific oligonucleotide
40	Tor 5' neg	FMT specific oligonucleotide
41	*Fuchsia FMT* (3282).nt	cDNA nucleotide
42	*Fuchsia* FMT (3282).aa	cDNA translated sequence
43	*Fuchsia FMT* full (3289).nt	cDNA nucleotide
44	*Fuchsia* FMT full (3289).aa	cDNA translated sequence
45	*Bam*HI linker	oligonucleotide linker
46	*Asc*II linker	oligonucleotide linker
47	*Sal*I linker	oligonucleotide linker

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B are schematic representations of the biosynthesis pathway for the flavonoid pigments in petunia. Enzymes involved in the pathway have been indicated as follows: PAL = Phenylalanine ammonia-lyase; C4H = Cinnamate 4-hydroxylase; 4CL = 4-coumarate: CoA ligase; CHS = Chalcone synthase; CHI = Chalcone flavanone isomerase; F3H = Flavanone 3-hydroxylase; DFR = Dihydroflavonol 4-reductase; ANS = Anthocyanidin synthase, 3GT= UDP-glucose: flavonoid 3-*O*-glucosyltransferase; 3RT = UDP rhamnose: anthocyanidin 3-glucoside rhamnosyltransferase, AR-AT = Anthocyanidin rutinoside acyltransferase, 5GT = Anthocyanin 5-glucosyltransferase; 3' FMT = Flavonoid 3' *O*-methyltransferase, 3'5' FMT = Flavonoid 3', 5' *O* - methyltransferase. Other abbreviations include: DHK = dihydrokaempferol, DHQ = dihydroquercetin, DHM = dihydromyricetin, P 3-G = pelargonidin 3-glucoside. Some of the genetic loci that control these reactions in petunia are shown in italics alongside the enzymes. Myricetin and pelargonidin based pigments occur rarely in petunia.

Figure 2 is a diagrammatic representation of the plasmid pCGP1903 containing the *difE* cDNA clone from *P. hybrida cv. V26.* ^{32}P-labelled fragments of the 0.9 kb *Eco*RI/*Xho*I fragment were used to probe the Old Glory Blue petal cDNA library. Abbreviations are as follows: Amp = the ampicillin resistance gene which confers resistance to the antibiotic ampicillin, f1 ori (+) = f1 filamentous phage origin of replication, ColE1ori = plasmid origin of replication, rev = approximate location of the M13 reverse primer site used in sequence analysis, -20 = approximate location of the M13 -20 primer site used in sequence analysis. Selected restriction enzyme sites are also marked.

Figure 3 is a diagrammatic representation of the plasmid pCGP1907 containing the *E20* cDNA clone from *P. hybrida cv. OGB.* Abbreviations are as follows: Amp = the ampicillin resistance gene which confers resistance to the antibiotic ampicillin, f1 ori (+) = f1 filamentous phage origin of replication, ori = plasmid origin of replication, rev = approximate location of the M13 reverse primer site used in sequence analysis, -20 =

approximate location of the M13 -20 primer site used in sequence analysis. Selected restriction enzyme sites are also marked.

Figure 4 is a diagrammatic representation of the plasmid pCGP1908 containing the *E33* cDNA clone from *P. hybrida cv. OGB.* Abbreviations are as follows: Amp = the ampicillin resistance gene which confers resistance to the antibiotic ampicillin, f1 ori (+) = f1 filamentous phage origin of replication, ori = plasmid origin of replication, rev = approximate location of the M13 reverse primer site used in sequence analysis, -20 = approximate location of the M13 -20 primer site used in sequence analysis. Selected restriction enzyme sites are also marked.

Figure 5 is a diagrammatic representation of the plasmid pCGP3086 (mut *E20* in pQE30) containing the mutated *E20* cDNA clone from *P. hybrida* in the bacterial expression vector pQE30. Abbreviations are as follows: Amp = the ampicillin resistance gene which confers resistance to the antibiotic ampicillin, Col E1 ori = *E. coli* plasmid origin of replication. Selected restriction enzyme sites are also marked.

Figure 6 is a diagrammatic representation of the binary plasmid pCGP1918. The chimaeric antisense *E20* gene from pCGP1910 was cloned into the binary vector pWTT2132 (DNAP) in a tandem orientation with the chimaeric *SuRB* gene. Abbreviations are as follows: TetR = tetracycline resistance gene which confers resistance to the antibiotic tetracycline; LB = left border; RB = right border; *SuRB* = the coding region and terminator sequence from the acetolactate synthase gene from tobacco; 35S = the promoter region from the cauliflower mosaic virus *(CaMV) 35S* gene, Mac = Hybrid promoter consisting of the promoter from the *mas* gene and a *CaMV 35S* enhancer region, mas 3' = the terminator region from the mannopine synthase gene of *Agrobacterium*; pVS1 = a broad host range origin of replication from a plasmid from *Pseuodomonas aeruginosa,* pACYC ori = modified replicon from pACYC184 from *E. coli.* Selected restriction enzyme sites are also marked.

Figure 7 is a diagrammatic representation of the binary plasmid pCGP1919. The chimaeric antisense *E33* gene from pCGP1911 was cloned into the binary vector pWTT2132 (DNAP) in a tandem orientation with the chimaeric *SuRB* gene. Abbreviations are as follows: TetR = the tetracycline resistance gene which confers resistance to the antibiotic tetracycline; LB = left border; RB = right border; *SuRB* = the coding region and terminator sequence from the acetolactate synthase gene from tobacco; 35S = the promoter region from the CaMV 35S gene, Mac = Hybrid promoter consisting of the promoter from the *mas* gene and a *CaMV 35S* enhancer region, mas 3' = the terminator region from the mannopine synthase gene of *Agrobacterium*; pVS1 = a broad host range origin of replication from a plasmid from *Pseuodomonas aeruginosa,* pACYC ori = modified replicon from pACYC184 from *E. coli*. Selected restriction enzyme sites are also marked.

Figure 8 is a diagrammatic representation of the plasmid pTMT5 containing the *TFMT* cDNA clone from *Torenia.* Abbreviations are as follows: Amp = the ampicillin resistance gene which confers resistance to the antibiotic ampicillin, f1 ori (+) = f1 filamentous phage origin of replication, ori = plasmid origin of replication, rev = approximate location of the M13 reverse primer site used in sequence analysis, -20 = approximate location of the M13 -20 primer site used in sequence analysis. Selected restriction enzyme sites are also marked.

Figure 9 is a diagrammatic representation of the plasmid pCGP3090 (mut *TFMT* in pQE30) containing the mutated *TFMT* cDNA clone from *Torenia* in the bacterial expression vector pQE30. Abbreviations are as follows: Amp = the ampicillin resistance gene which confers resistance to the antibiotic ampicillin, Col E1 ori = *E. coli* plasmid origin of replication. Selected restriction enzyme sites are also marked.

Figure 10 is a diagrammatic representation of the plasmid pCGP3097. The *Torenia FMT* clone (*TFMT*) from pTMT5 was cloned into a *CaMV35S* expression cassette. Abbreviations are as follows: Amp = the ampicillin resistance gene which confers resistance to the antibiotic ampicillin, 35S 5' = the promoter region from the *CaMV 35S*

gene, 35S 3' = the terminator region from the *CaMV 35S* gene. Selected restriction enzyme sites are also marked.

Figure 11 is a diagrammatic representation of the binary plasmid pCGP3099. The chimaeric *Torenia FMT* gene (*TFMT*) from pCGP3097 (Figure 10) was cloned into the binary vector pCGP1988 (Figure 12) in a tandem orientation with the chimaeric *SuRB* gene. Abbreviations are as follows: TetR= the tetracycline resistance gene which confers resistance to the antibiotic tetracycline; LB = left border; RB = right border; *SuRB* = the coding region and terminator sequence from the acetolactate synthase gene from tobacco; 35S 5' = the promoter region from the *CaMV 35S* gene, 35S 3' = the terminator region from the *CaMV 35S* gene, pVS1 = a broad host range origin of replication from a plasmid from *Pseuodomonas aeruginosa,* pACYC ori = modified replicon from pACYC184 from *E. coli*. Selected restriction enzyme sites are also marked.

Figure 12 is a diagrammatic representation of the binary plasmid pCGP1988. The multi-cloning site of the binary vector pWTT2132 (DNAP) was replaced with the multi-cloning site from pNEB193 (New England Biolabs). Abbreviations are as follows: TetR= the tetracycline resistance gene which confers resistance to the antibiotic tetracycline; LB = left border; RB = right border; *SuRB* = the coding region and terminator sequence from the acetolactate synthase gene from tobacco; 35S 5' = the promoter region from the *CaMV 35S* gene, pVS1 = a broad host range origin of replication from a plasmid from *Pseuodomonas aeruginosa,* pACYC ori = modified replicon from pACYC184 from *E. coli*. Selected restriction enzyme sites are also marked.

Figure 13 is a diagrammatic representation of the binary plasmid pCGP3254. The chimaeric *F3'5'H* gene from pCGP2092 (Figure 14) was cloned into the binary plasmid pCGP3099 (Figure 11) in a tandem orientation with the chimaeric *SuRB* gene and the chimaeric *TFMT* gene. Abbreviations are as follows: *F3'5'H* = flavonoid 3', 5' hydroxylase cDNA clone from *Viola*, *TFMT* = *Torenia* FMT cDNA clone, TetR = the tetracycline resistance gene which confers resistance to the antibiotic tetracycline; LB = left border; RB = right border; *SuRB* = the coding region and terminator sequence from the

acetolactate synthase gene from tobacco; 35S 5' = the promoter region from the *CaMV 35S* gene, 35S 3' = the terminator region from the *CaMV 35S* gene, pVS1 = a broad host range origin of replication from a plasmid from *Pseuodomonas aeruginosa,* pACYC ori = modified replicon from pACYC184 from *E. coli*. Selected restriction enzyme sites are also marked.

Figure 14 is a diagrammatic representation of the plasmid pCGP2092. The *Viola F3'5'H* clone from pCGP1961 was cloned into a *CaMV35S* expression cassette. Abbreviations are as follows: *F3'5'H* = flavonoid 3', 5' hydroxylase cDNA clone from *Viola*, Amp = the ampicillin resistance gene which confers resistance to the antibiotic ampicillin, 35S 5' = the promoter region from the *CaMV 35S* gene, 35S 3' = the terminator region from the *CaMV 35S* gene. Selected restriction enzyme sites are also marked.

Figure 15 is a diagrammatic representation of the binary plasmid pSPB1534. The chimaeric *Viola F3'5'H* gene from pSPB580 (Figure 16) was cloned in a tandem orientation with the chimaeric Petunia *FMT* gene and the selectable marker gene of the Ti binary plasmid pSPB1531 (Figure 17). Abbreviations are as follows: *F3'5'H* = flavonoid 3', 5' hydroxylase cDNA clone from *Viola*, *PFMT* = *Petunia FMT* cDNA clone *nptIII* = the neomycin phosphotransferase III gene which confers resistance to the antibiotic kanamycin, *nptII* = the neomycin phosphotransferase II gene which confers resistance to the antibiotic kanamycin, e35S 5' = an enhanced promoter region from the *CaMV 35S* gene, petD8 3' = the terminator region from the *Petunia PLTP* gene, nos 5' = promoter region from the nopaline synthase gene of *Agrobacterium*, nos 3' = terminator region from the nopaline synthase gene of *Agrobacterium*, ColE1 = *E. coli* plasmid ColE1 origin, RK2 = broad host range Gram-negative plasmid RK2 origin, LB = left border, RB = right border. Selected restriction enzyme sites are also marked.

Figure 16 is a diagrammatic representation of the plasmid pSPB580. The *Viola F3'5'H* (B*P#40*) cDNA clone (from pCGP1961) was cloned between an enhanced *CaMV 35S* promoter fragment (from pBE2113-GUS) and a *Petunia PLTP (D8)* terminator fragment (from pCGP13ΔBam). Abbreviations are as follows: *F3'5'H* = flavonoid 3', 5'

hydroxylase cDNA clone from *Viola*, Amp = the ampicillin resistance gene which confers resistance to the antibiotic ampicillin, e35S 5' = an enhanced promoter region from the *CaMV 35S* gene, ColE1 = *E. coli* plasmid ColE1 origin, petD8 3' = the terminator region from the *Petunia PLTP* gene.

Figure 17 is a diagrammatic representation of the binary plasmid pSPB1531. The chimaeric *Petunia FMT* (*PFMT*) cDNA clone was amplified by PCR (from pCGP1907) (Figure 3) and replaced the *GUS* coding region of the binary plasmid pSPB176 (Figure 20). Abbreviations are as follows: *nptIII* = the neomycin phosphotransferase III gene which confers resistance to the antibiotic kanamycin, *nptII* = the neomycin phosphotransferase II gene which confers resistance to the antibiotic kanamycin, e35S 5' = an enhanced promoter region from the *CaMV 35S* gene, nos 5' = promoter region from the nopaline synthase gene of *Agrobacterium*, nos 3' = terminator region from the nopaline synthase gene of *Agrobacterium*, ColE1 = *E. coli* plasmid ColE1 origin, RK2 = broad host range Gram-negative plasmid RK2 origin, LB = left border, RB = right border. Selected restriction enzyme sites are also marked.

Figure 18 is a diagrammatic representation of the binary plasmid pSPB1532. The chimaeric *Viola F3'5'H* gene from pSPB580 (Figure 16) was cloned in a tandem orientation with the chimaeric *Petunia FMT* gene and the selectable marker gene of the Ti binary plasmid pSPB1531 (Figure 17). Abbreviations are as follows: *F3'5'H* = flavonoid 3', 5' hydroxylase cDNA clone from *Viola*, *TFMT* = *Torenia* FMT cDNA clone *nptIII* = the neomycin phosphotransferase III gene which confers resistance to the antibiotic kanamycin, *nptII* = the neomycin phosphotransferase II gene which confers resistance to the antibiotic kanamycin, e35S 5' = an enhanced promoter region from the *CaMV 35S* gene, petD8 3' = the terminator region from the *Petunia PLTP* gene, nos 5' = promoter region from the nopaline synthase gene of *Agrobacterium*, nos 3' = terminator region from the nopaline synthase gene of *Agrobacterium*, ColE1 = *E. coli* plasmid ColE1 origin, RK2 = broad host range Gram-negative plasmid RK2 origin, LB = left border, RB = right border. Selected restriction enzyme sites are also marked.

Figure 19 is a diagrammatic representation of the binary plasmid pSPB1530. The chimaeric *Torenia FMT* (*TFMT*) cDNA clone was amplified by PCR (from pTMT5) (Figure 8) and replaced the *GUS* coding region of the binary plasmid pSPB176 (Figure 20). Abbreviations are as follows: *nptIII* = the neomycin phosphotransferase III gene which confers resistance to the antibiotic kanamycin, *nptII* = the neomycin phosphotransferase II gene which confers resistance to the antibiotic kanamycin, e35S 5' = an enhanced promoter region from the *CaMV 35S* gene, nos 5' = promoter region from the nopaline synthase gene of *Agrobacterium*, nos 3' = terminator region from the nopaline synthase gene of *Agrobacterium*, ColE1 = *E. coli* plasmid ColE1 origin, RK2 = broad host range Gram-negative plasmid RK2 origin, LB = left border, RB = right border. Selected restriction enzyme sites are also marked.

Figure 20 is a diagrammatic representation of the binary plasmid pSPB176. A chimaeric *GUS* gene (from pBE2113-GUS) was cloned in a tandem orientation to the *nptII* selectable marker gene of the Ti binary vector pBINPlus. Abbreviations are as follows: *nptIII* = the neomycin phosphotransferase III gene which confers resistance to the antibiotic kanamycin, *nptII* = the neomycin phosphotransferase II gene which confers resistance to the antibiotic kanamycin, e35S 5' = an enhanced promoter region from the *CaMV 35S* gene, petD8 3' = the terminator region from the *Petunia PLTP* gene, nos 5' = promoter region from the nopaline synthase gene of *Agrobacterium*, nos 3' = terminator region from the nopaline synthase gene of *Agrobacterium*, ColE1 = *E. coli* plasmid ColE1 origin, RK2 = broad host range Gram-negative plasmid RK2 origin, LB = left border, RB = right border. Selected restriction enzyme sites are also marked.

Figure 21 is a diagrammatic representation of the plasmid pCGP3267. A partial clone of *Fuchsia FMT* was amplified using PCR and single stranded cDNA (prepared from total RNA isolated from *Fuchsia* petals) as template and cloned into the plasmid pCR2.1. Abbreviations are as follows: Amp = the ampicillin resistance gene which confers resistance to the antibiotic tetracycline, Kan = the kanamycin resistance gene which confers resistance to the antibiotic kanamycin, f1 ori (+) = f1 filamentous phage origin of replication, ColE1 ori = plasmid origin of replication, rev = approximate location of the

M13 reverse primer site used in sequence analysis, -21 = approximate location of the M13 -21 primer site used in sequence analysis. Selected restriction enzyme sites are also marked.

Figure 22 is a diagrammatic representation of the plasmid pCGP3289. A full-length version of *Fuchsia FMT* (*Fuchsia FMT full*) was cloned into the plasmid pCR2.1. Abbreviations are as follows: Amp = the ampicillin resistance gene which confers resistance to the antibiotic ampicillin, Kan = the kanamycin resistance gene which confers resistance to the antibiotic kanamycin, f1 ori (+) = f1 filamentous phage origin of replication, ColE1 ori = plasmid origin of replication, rev = approximate location of the M13 reverse primer site used in sequence analysis, -21 = approximate location of the M13 -21 primer site used in sequence analysis. Selected restriction enzyme sites are also marked.

Figure 23 is a diagrammatic representation of the plasmid pCGP3290. The *Fuchsia FMT* cDNA clone (*FFMT*) from pCGP3289 (Figure 22) was cloned into a *CaMV 35S* expression cassette. Abbreviations are as follows: Amp = Amp = the ampicillin resistance gene which confers resistance to the antibiotic ampicillin, 35S 5' = the promoter region from the *CaMV 35S* gene, 35S 3' = the terminator region from the *CaMV 35S* gene. Selected restriction enzyme sites are also marked.

Figure 24 is a diagrammatic representation of the binary plasmid pCGP2788. The *35S 5': Torenia FMT: 35S 3'* expression cassette was removed from the binary plasmid pCGP3254 (Figure 13) to leave a binary vector with the *35S 5': Viola F3'5'H: 35S 3'* expression cassette in tandem with the *35S 5': SuRB selectable marker gene.* Abbreviations are as follows: *F3'5'H* = flavonoid 3', 5' hydroxylase cDNA clone from *Viola*, TetR = the tetracycline resistance gene which confers resistance to the antibiotic tetracycline; LB = left border; RB = right border; *SuRB* = the coding region and terminator sequence from the acetolactate synthase gene from tobacco; 35S 5' = the promoter region from the *CaMV 35S* gene, 35S 3' = the terminator region from the *CaMV 35S* gene, pVS1 = a broad host range origin of replication from a plasmid from *Pseuodomonas aeruginosa,*

pACYC ori = modified replicon from pACYC184 from *E. coli*. Selected restriction enzyme sites are also marked.

Figure 25 is a diagrammatic representation of the binary plasmid pCGP3292. The *35S 5': FFMT: 35S 3'* expression cassette from pCGP3290 (Figure 23) was cloned in a tandem orientation to the *35S 5': SuRB* and *35S 5': F3'5'H: 35S 3'* expression cassettes of the Ti binary plasmid pCGP2788 (Figure 24). Abbreviations are as follows: *F3'5'H* = flavonoid 3', 5' hydroxylase cDNA clone from *Viola*, *FFMT* = *Fuchsia FMT* cDNA clone, TetR = the tetracycline resistance gene which confers resistance to the antibiotic tetracycline; LB = left border; RB = right border; *SuRB* = the coding region and terminator sequence from the acetolactate synthase gene from tobacco; 35S 5' = the promoter region from the *CaMV 35S* gene, 35S 3' = the terminator region from the *CaMV 35S* gene, pVS1 = a broad host range origin of replication from a plasmid from *Pseuodomonas aeruginosa*, pACYC ori = modified replicon from pACYC184 from *E. coli*. Selected restriction enzyme sites are also marked.

Figure 26 shows a dendogram that illustrates the clustering relationship between deduced amino acid sequences of petunia (pCGP1907.aa), *Torenia* (pTMT5.aa) and *Fuchsia* (pCGP3267.aa) FMTs with other full length plant *O*-methyltransferases (OMT) of both Class I and Class II found in the GenBank database. The Genbank accession numbers of each SAM-OMT in the database are shown in brackets.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with the present invention, a genetic sequence encoding a methyltransferase and, more particularly, a flavonoid methyltransferase (hereinafter referred to as "FMT") has been identified and cloned. The recombinant sequence permits the modulation of methyltransferase when it is attached to a flavonoid molecule. Substrates include anthocyanins with a hydroxyl group attached to the molecule such as anthocyanins based on the anthocyanidins delphinidin, cyanidin and petunidin including but not limited to delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, delphinidin 3, 5-diglucoside, cyanidin 3, 5-diglucoside, petunidin 3, 5-diglucoside thereby providing a means to manipulate petal color. Accordingly, the present invention relates to the altering of FMT activity in plants, which encompasses elevating or reducing (i.e. modulating) levels of existing FMT activity by introducing a sequence of the present invention. Reduction in levels of FMT activity may also be referred to as down-regulation. Moreover, the present invention extends to plants and reproductive or vegetative parts thereof including flowers, seeds, vegetables, leaves, stems, etc., and more particularly, genetically modified or ornamental transgenic plants.

A "transgenic plant" includes any genetically modified plant and the terms "transgenic" and "genetically modified" may be used interchangeably through the subject specification.

Accordingly, one aspect of the present invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding FMT or a functional derivative of the enzyme.

The present invention is described and exemplified herein by reference to the identification, cloning and manipulation of genetic sequences encoding FMT which, up to the present time, is a particularly convenient and useful flavonoid methyltransferase enzyme for the practice of the invention herein disclosed. This is done, however, with the understanding that the present invention extends to all novel FMT enzymes and their functional derivatives.

For convenience and by way of short hand notation only, reference herein to a flavonoid methylating enzyme includes FMTs acting on flavonoids such as anthocyanins, flavonols and/or flavones. Preferably, the flavonoid methylating enzyme is FMT. The FMT enzyme may also be considered to include a polypeptide or protein having FMT activity or FMT-like activity. The latter encompasses derivatives having altered FMT activities.

A preferred aspect of the present invention, therefore, is directed to an isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding FMT or a functional mutant, derivative, part, fragment, homolog or analog of FMT.

By the term "nucleic acid molecule" is meant a genetic sequence in a non-naturally occurring condition. Generally, this means isolated away from its natural state or synthesized or derived in a non-naturally-occurring environment. More specifically, it includes nucleic acid molecules formed or maintained *in vitro,* including genomic DNA fragments recombinant or synthetic molecules and nucleic acids in combination with heterologous nucleic acids. It also extends to the genomic DNA or cDNA or part thereof encoding FMT or a part thereof in reverse orientation relative to its own or another promoter. It further extends to naturally occurring sequences following at least a partial purification relative to other nucleic acid sequences.

The term "genetic sequences" is used herein in its most general sense and encompasses any contiguous series of nucleotide bases specifying directly, or *via* a complementary series of bases, a sequence of amino acids in an FMT enzyme. Such a sequence of amino acids may constitute a partial FMT such as set forth in SEQ ID NO:22 or SEQ ID NO:42 or a full-length FMT such as is set forth in SEQ ID NO:2 or SEQ ID NO:5 or SEQ ID NO:7 or SEQ ID NO:12 or SEQ ID NO:44 or an active truncated form thereof or may correspond to a particular region such as an N-terminal, C-terminal or internal portion of the enzyme. A genetic sequence may also be referred to as a sequence of nucleotides or a nucleotide sequence and include a recombinant fusion of two or more sequences.

The genetic sequence of the present invention may also be subject to modified codon useage to improve or otherwise facilitate expression in a particular host cell.

In accordance with the above aspects of the present invention there is provided a nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:1 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:11 or SEQ ID NO:21 or SEQ ID NO:26 SEQ ID NO:41 or SEQ ID NO:43 or having at least about 50% similarity thereto or capable of hybridizing to the sequence set forth in SEQ ID NO:1 under low stringency conditions.

Alternative percentage similarity encompassed by the present invention include at least about 60% or at least about 70% or at least about 80% or at least about 90% or above, such as about 95% or about 96% or about 97% or about 98% or about 99%.

In a particularly preferred embodiment, there is provided an isolated nucleic acid molecule comprising a nucleotide sequence or complementary nucleotide sequence substantially as set forth in SEQ ID NO:1 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:11 or SEQ ID NO:21 or SEQ ID NO:26 or SEQ ID NO:41 or SEQ ID NO:43 having at least about 50% similarity thereto or capable of hybridising to the sequence set forth in SEQ ID NO:1 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:11 or SEQ ID NO:21 or SEQ ID NO:26 or SEQ ID NO:41 or SEQ ID NO:43 or complementary strands of either under low stringency conditions, wherein said nucleotide sequence encodes a polypeptide having FMT activity.

For the purposes of determining the level of stringency to define nucleic acid molecules capable of hybridizing to SEQ ID NO:1 or SEQ ID NO: 4 or SEQ ID NO: 6 or SEQ ID NO: 11 or SEQ ID NO: 21 or SEQ ID NO: 26 or SEQ ID NO:41 or SEQ ID NO:43 reference herein to a low stringency includes and encompasses from at least about 0% to at least about 15% v/v formamide and from at least about 1 M to at least about 2 M salt for hybridization, and at least about 1 M to at least about 2 M salt for washing conditions.

Generally, low stringency is from about 25-30°C to about 42°C. The temperature may be altered and higher temperatures used to replace formamide and/or to give alternative stringency conditions. Alternative stringency conditions may be applied where necessary, such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5 M to at least about 0.9 M salt for hybridization, and at least about 0.5 M to at least about 0.9 M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01 M to at least about 0.15 M salt for hybridization, and at least about 0.01 M to at least about 0.15 M salt for washing conditions. In general, washing is carried out $T_m = 69.3 + 0.41$ (G+C)% (Marmur and Doty, *J. Mol. Biol. 5*:109, 1962). However, the T_m of a duplex DNA decreases by 1°C with every increase of 1% in the number of mismatch base pairs (Bonner and Laskey, *Eur. J. Biochem. 46*: 83, 1974). Formamide is optional in these hybridization conditions. Accordingly, particularly preferred levels of stringency are defined as follows: low stringency is 6 x SSC buffer, 1.0% w/v SDS at 25-42°C; a moderate stringency is 2 x SSC buffer, 1.0% w/v SDS at a temperature in the range 20°C to 65°C; high stringency is 0.1 x SSC buffer, 0.1% w/v SDS at a temperature of at least 65°C.

Another aspect of the present invention provides a nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in SEQ ID NO:2 or SEQ ID NO:5 or SEQ ID NO:7 or SEQ ID NO:12 or SEQ ID NO:22 or SEQ ID NO:42 or SEQ ID NO:44 or an amino acid sequence having at least about 50% similarity thereto.

The term similarity as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, similarity includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, similarity includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particularly preferred embodiment,

nucleotide and sequence comparisons are made at the level of identity rather than similarity.

Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence", "comparison window", "sequence similarity", "sequence identity", "percentage of sequence similarity", "percentage of sequence identity", "substantially similar" and "substantial identity". A "reference sequence" is at least 12 but frequently 15 to 18 and often at least 25 or above, such as 30 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides may each comprise (1) a sequence (i.e. only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window" refers to a conceptual segment of typically 12 contiguous residues that is compared to a reference sequence. The comparison window may comprise additions or deletions (i.e. gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (i.e. resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as, for example, disclosed by Altschul *et al.* (*Nucl. Acids Res. 25:* 3389, 1997). A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.* (Current Protocols in Molecular Biology, John Wiley & Sons Inc, 1994-1998, Chapter 15, 1998).

The terms "sequence similarity" and "sequence identity" as used herein refers to the extent that sequences are identical or functionally or structurally similar on a nucleotide-by-

nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity", for example, is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g. A, T, C, G, I) or the identical amino acid residue (e.g. Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. For the purposes of the present invention, "sequence identity" will be understood to mean the "match percentage" calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA) using standard defaults as used in the reference manual accompanying the software. Similar comments apply in relation to sequence similarity.

The nucleic acid sequences contemplated herein also encompass oligonucleotides useful as genetic probes for amplification reactions or as antisense or sense molecules capable of regulating expression of the corresponding gene in a plant. An antisense molecule as used herein may also encompass a genetic construct comprising the structural genomic or cDNA gene or part thereof in reverse orientation relative to its or another promoter. It may also encompass a homologous genetic sequence. An antisense or sense molecule may also be directed to terminal or internal portions of the gene encoding a polypeptide having FMT activity or to combinations of the above such that the expression of the gene is reduced or eliminated.

With respect to this aspect of the invention, there is provided an oligonucleotide of 5-50 nucleotides having substantial similarity or complementarity to a part or region of a molecule with a nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:11 or SEQ ID NO:21 or SEQ ID NO:26 or SEQ ID NO:41 or SEQ ID NO:43 or a complementary form thereof. By substantial similarity or complementarity in this context is meant a hybridizable similarity under low, alternatively

and preferably medium and alternatively and most preferably high stringency conditions specific for oligonucleotide hybridization (Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual.* (2nd edition), Cold Spring Harbor Laboratory Press, USA, 1989). Such an oligonucleotide is useful, for example, in screening *FMT* genetic sequences from various sources or for monitoring an introduced genetic sequence in a transgenic plant. The preferred oligonucleotide is directed to a conserved *FMT* genetic sequence or a sequence conserved within a plant genus, plant species and/or plant variety.

In one aspect of the present invention, the oligonucleotide corresponds to the 5' or the 3' end of the *FMT* genetic sequence. For convenience, the 5' end is considered herein to define a region substantially between the start codon of the structural gene to a centre portion of the gene, and the 3' end is considered herein to define a region substantially between the centre portion of the gene and the terminating codon of the structural gene. It is clear, therefore, that oligonucleotides or probes may hybridize to the 5' end or the 3' end or to a region common to both the 5' and the 3' ends. The present invention extends to all such probes.

In one embodiment, the nucleic acid sequence encoding an FMT or various functional derivatives thereof is used to reduce the level of an endogenous *FMT* (e.g. *via* co-suppression) or other post transcriptional gene silencing (PTGS) processes including RNAi or alternatively the nucleic acid sequence encoding this enzyme or various derivatives or parts thereof is used in the antisense orientation to reduce the level of FMT. The use of sense strands, double or partially single stranded such as constructs with hairpin loops is particularly useful in inducing a PTGS response. In a further alternative, ribozymes could be used to inactivate target nucleic acid sequences.

Still a further embodiment encompasses post-transcriptional inhibition to reduce translation into polypeptide material.

Reference herein to the altering of FMT activity relates to an elevation or reduction in activity of up to 30% or more preferably of 30-50%, or even more preferably 50-75% or

still more preferably 75% or greater above or below the normal endogenous or existing levels of activity. Such elevation or reduction may be referred to as modulation of FMT enzyme activity. Generally, modulation is at the level of transcription or translation of *FMT* genetic sequences.

The nucleic acids of the present invention may be a ribonucleic acid or deoxyribonucleic acids, single or double stranded and linear or covalently closed circular molecules. Preferably, the nucleic acid molecule is cDNA. The present invention also extends to other nucleic acid molecules which hybridize under low, preferably under medium and most preferably under high stringency conditions with the nucleic acid molecules of the present invention and in particular to the sequence of nucleotides set forth in SEQ ID NO:1 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:11 or SEQ ID NO:21 or SEQ ID NO:26 or SEQ ID NO:41 or SEQ ID NO:43 or a part or region thereof. In its most preferred embodiment, the present invention extends to a nucleic acid molecule having a nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:11 or SEQ ID NO:21 or SEQ ID NO:26 or SEQ ID NO:41 or SEQ ID NO:43 or to a molecule having at least 40%, more preferably at least 45%, even more preferably at least 55%, still more preferably at least 65%-70%, and yet even more preferably greater than 85% similarity at the level of nucleotide or amino acid sequence to at least one or more regions of the sequence set forth in SEQ ID NO:1 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:11 or SEQ ID NO:21 or SEQ ID NO:26 or SEQ ID NO:41 or SEQ ID NO:43 and wherein the nucleic acid encodes or is complementary to a sequence which encodes an enzyme having FMT activity. It should be noted, however, that nucleotide or amino acid sequences may have similarities below the above given percentages and yet still encode FMT activity and such molecules may still be considered in the scope of the present invention where they have regions of sequence conservation. The present invention further extends to nucleic acid molecules in the form of oligonucleotide primers or probes capable of hybridizing to a portion of the nucleic acid molecules contemplated above, and in particular those set forth in SEQ ID NO:1 and/or SEQ ID NO:4 and/or SEQ ID NO:6 and/or SEQ ID NO:11 and/or SEQ ID NO:21 and/or SEQ ID NO:26 and/or SEQ ID NO:41 and/or SEQ ID NO:43, under low, preferably under medium and most preferably

under high stringency conditions. Preferably the portion corresponds to the 5' or the 3' end of the gene. For convenience the 5' end is considered herein to define a region substantially between the start codon of the structural genetic sequence to a centre portion of the gene, and the 3' end is considered herein to define a region substantially between the centre portion of the gene and the terminating codon of the structural genetic sequence. It is clear, therefore, that oligonucleotides or probes may hybridize to the 5' end or the 3' end or to a region common to both the 5' and the 3' ends. The present invention extends to all such probes.

The term gene is used in its broadest sense and includes cDNA corresponding to the exons of a gene. Accordingly, reference herein to a gene is to be taken to include:-

(i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5'- and 3'- untranslated sequences); or

(ii) mRNA or cDNA corresponding to the coding regions (i.e. exons) and 5'- and 3'- untranslated sequences of the gene.

The term "gene" is also used to describe synthetic or fusion molecules encoding all or part of an expression product. In particular embodiments, the term "nucleic acid molecule" and "gene" may be used interchangeably.

The nucleic acid or its complementary form may encode the full-length enzyme or a part or derivative thereof. By "derivative" is meant any single or multiple amino acid substitutions, deletions, and/or additions relative to the naturally occurring enzyme and which retains FMT activity. In this regard, the nucleic acid includes the naturally occurring nucleotide sequence encoding FMT or may contain single or multiple nucleotide substitutions, deletions and/or additions to said naturally occurring sequence. The nucleic acid of the present invention or its complementary form may also encode a "part" of the FMT, whether active or inactive, and such a nucleic acid molecule may be useful as an

oligonucleotide probe, primer for polymerase chain reactions or in various mutagenic techniques, or for the generation of antisense molecules.

Reference herein to a "part" of a nucleic acid molecule, nucleotide sequence or amino acid sequence, preferably relates to a molecule which contains at least about 10 contiguous nucleotides or five contiguous amino acids, as appropriate.

Amino acid insertional derivatives of the FMT of the present invention include amino and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterized by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Typical substitutions are those made in accordance with Table 2.

TABLE 2

Suitable residues for amino acid substitutions

ORIGINAL RESIDUE	EXEMPLARY SUBSTITUTIONS
Ala	Ser
Arg	Lys
Asn	Gln; His
Asp	Glu
Cys	Ser
Gln	Asn; Glu
Glu	Asp
Gly	Pro

ORIGINAL RESIDUE	EXEMPLARY SUBSTITUTIONS
His	Asn; Gln
Ile	Leu; Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile; Val
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu; Met

Where the FMT is derivatized by amino acid substitution, the amino acids are generally replaced by other amino acids having like properties, such as hydrophobicity, hydrophilicity, electronegativity, bulky side chains and the like. Amino acid substitutions are typically of single residues. Amino acid insertions will usually be in the order of about 1-10 amino acid residues and deletions will range from about 1-20 residues. Preferably, deletions or insertions are made in adjacent pairs, i.e. a deletion of two residues or insertion of two residues.

The amino acid variants referred to above may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis (Merrifield, *J. Am. Chem. Soc. 85:* 2149, 1964) and the like, or by recombinant DNA manipulations. Techniques for making substitution mutations at predetermined sites in DNA having known or partially known sequence are well known and include, for example, M13 mutagenesis. The manipulation of DNA sequence to produce variant proteins which manifest as substitutional, insertional or deletional variants are conveniently described, for example, in Sambrook *et al.*, (1989), *supra.*

Other examples of recombinant or synthetic mutants and derivatives of the FMT enzyme of the present invention include single or multiple substitutions, deletions and/or additions of any molecule associated with the enzyme such as carbohydrates, lipids and/or proteins or polypeptides.

The terms "analogs" and "derivatives" also extend to any functional chemical equivalent of FMT and also to any amino acid derivative described above. For convenience, reference to FMT herein includes reference to any functional mutant, derivative, part, fragment, homolog or analog thereof.

The present invention is exemplified using nucleic acid sequences derived from *Petunia*, *Torenia* or *Fuchsia* since this represents the most convenient and preferred source of material to date. However, one skilled in the art will immediately appreciate that similar sequences can be isolated from any number of sources such as other plants or certain microorganisms. All such nucleic acid sequences encoding directly or indirectly an FMT are encompassed by the present invention regardless of their source. Examples of other suitable sources of genes encoding FMTs include, but are not limited to *Petunia sp.*, *Plumbago sp.*, *Vitis sp.*, *Babiana stricta*, *Pinus sp.*, *Picea sp.*, *Larix sp.*, *Phaseolus sp.*, *Solanum sp.*, *Vaccinium sp.*, *Cyclamen sp.*, *Iris sp.*, *Pelargonium* sp., *Geranium sp.*, *Pisum sp.*, *Lathyrus sp.*, *Clitoria sp.*, *Catharanthus sp.*, *Malvia sp.*, *Mucuna sp.*, *Vicia sp.*, *Saintpaulia sp.*, *Lagerstroemia sp.*, *Tibouchina sp.*, *Hypocalyptus sp.*, *Rhododendron sp.*, *Linum sp.*, *Macroptilium sp.*, *Hibiscus sp.*, *Hydrangea sp.*, *Ipomoea sp.*, *Cymbidium sp.*, *Millettia sp.*, *Hedysarum sp.*, *Lespedeza sp.*, *Antigonon sp.*, *Pisum sp.*, etc.

In accordance with the present invention, a nucleic acid sequence encoding FMT may be introduced into and expressed in a transgenic plant in either orientation thereby providing a means either to convert suitable substrates, if synthesized in the plant cell, ultimately into peonidin, petunidin or malvidin derivatives or other methyl-flavonoids, or alternatively to inhibit such conversion of metabolites by reducing or eliminating endogenous or existing FMT activity. The production of these anthocyanins or other flavonoids will modify petal color and may contribute to the production of a bluer color. Expression of the nucleic acid

sequence in the plant may be constitutive, inducible or developmental and may also be tissue-specific. The word "expression" is used in its broadest sense to include production of RNA or of both RNA and protein. It also extends to partial expression of a nucleic acid molecule.

The terms "genetically modified plant" and "transgenic plant" refer to any plant or progeny or subsequent offspring therefrom or vegetatively propagated new plant that has become transformed after the introduction of novel nucleic acid sequence using appropriate molecular biology techniques. The two terms are used interchangeably through out the specification. The nucleic acid sequence may be derived from the same or a different species of plant to that which is being transformed. It is contemplated that the nucleic acid could encode a polypeptide or be complementary to a sequence encoding a polypeptide or a mutant, derivative, part, fragment or portion thereof. Alternatively the nucleic acid sequence may be from the non-coding region of a genome.

The genetically modified or transgenic plants of the present invention include horticultural and agricultural species.

The term "horticultural plant species" includes but is not limited to floricultural plants (for example, cut-flowers, potted flowering plants), ornamental plants (for example, ornamental foliage plants) and all other forms of horticulture (such as, bedding plants, pot-plants, garden-plants).

The term "agricultural plant species" includes but is not limited to broad acre food and non-food crops (for example, wheat, corn, cotton, maize, pasture), fruit, nut and vegetable crops (for example apples, oranges, bananas, almonds, walnuts, macadamias, carrots, peas, potatoes, eggplants, grapes, tomatoes) and viticulture.

According to current understanding there will be some overlap between horticultural and agricultural plant species.

According to this aspect of the present invention, there is provided a method for producing a transgenic plant, such as but not limited to a transgenic flowering plant, capable of synthesizing FMT, said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence which comprises a sequence of nucleotides encoding said FMT under conditions permitting the eventual expression of said nucleic acid sequence, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence. The transgenic plant may thereby produce non-indigenous FMT at elevated levels relative to the amount expressed in a comparable non-transgenic plant.

Another aspect of the present invention contemplates a method for producing a transgenic plant with reduced indigenous or existing FMT activity, said method comprising stably transforming a cell of a suitable plant with a nucleic acid molecule which comprises a sequence of nucleotides encoding or complementary to a sequence encoding an FMT activity, regenerating a transgenic plant from the cell and where necessary growing said transgenic plant under conditions sufficient to permit the expression of the nucleic acid.

Yet another aspect of the present invention contemplates a method for producing a genetically modified plant with reduced indigenous or existing FMT activity, said method comprising altering the *FMT* gene through modification of the indigenous sequences *via* homologous recombination from an appropriately altered *FMT* gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

As used herein an "indigenous" enzyme is one, which is native to or naturally expressed in a particular cell. A "non-indigenous" enzyme is an enzyme not native to the cell but expressed through the introduction of genetic material into a plant cell; for example, through a transgene. An "endogenous" enzyme is an enzyme produced by a cell but which may or may not be indigenous to that cell.

In a preferred embodiment, the present invention contemplates a method for producing a transgenic plant, such as but not limited to a transgenic flowering plant, exhibiting altered inflorescence properties, said method comprising stably transforming a cell of a suitable plant with a nucleic acid sequence of the present invention, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence into an FMT. Alternatively, said method may comprise stably transforming a cell of a suitable plant with a nucleic acid sequence of the present invention or its complementary sequence, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to alter the level of activity of the indigenous or existing FMT. Preferably the altered level would be less than the indigenous or existing level of FMT activity in a comparable non-transgenic plant. Without wishing to limit the present invention, one theory of mode of action is that reduction of the indigenous FMT activity requires the expression of the introduced nucleic acid sequence or its complementary sequence. However, expression of the introduced genetic sequence or its complement may not be required to achieve the desired effect: namely, a flowering plant exhibiting altered inflorescence properties.

The term "inflorescence" as used herein refers to the flowering part of a plant. As indicated above, reference to a "transgenic plant" may also be read as a "genetically modified plant".

In a related embodiment, the present invention contemplates a method for producing a plant such as but not limited to a transgenic flowering plant exhibiting altered inflorescence properties, said method comprising alteration of the *FMT* gene through modification of the indigenous sequences *via* homologous recombination from an appropriately altered *FMT* gene or derivative or part thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

Preferably, the altered inflorescence includes the production of different shades of blue or red flowers or other colors, depending on the genotype and physiological conditions of the recipient plant.

Accordingly, the present invention extends to a method for producing a transgenic plant capable of expressing a recombinant gene encoding an FMT or part thereof or which carries a nucleic acid sequence which is substantially complementary to all or a part of a mRNA molecule optionally transcribable where required to effect regulation of an *FMT*, said method comprising stably transforming a cell of a suitable plant with the isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding, an FMT, where necessary under conditions permitting the eventual expression of said isolated nucleic acid molecule, and regenerating a transgenic plant from the cell. By "suitable plant" is meant a plant capable of producing anthocyanidin 3-glucosides and possessing the appropriate physiological properties required for the development of the color desired. Examples of suitable plants include but are not limited to *Torenia*, *Begonia*, *Cyclamen*, *Nierembergia*, *Catharanthus*, *Pelogonium*, *Orchid*, grape, *Euphorbia* or *Fuchsia*

One skilled in the art will immediately recognize the variations applicable to the methods of the present invention, such as increasing or decreasing the expression of the enzyme naturally present in a target plant leading to differing shades of colors such as different shades of blue, purple or red.

The present invention, therefore, extends to all transgenic plants or parts of transgenic plants or progeny of the transgenic plants containing all or part of the nucleic acid sequences of the present invention, or antisense forms thereof and/or any homologs or related forms thereof and, in particular, those transgenic plants which exhibit altered inflorescence properties. The transgenic plants may contain an introduced nucleic acid molecule comprising a nucleotide sequence encoding or complementary to a sequence encoding an FMT. Generally, the nucleic acid would be stably introduced into the plant genome, although the present invention also extends to the introduction of an *FMT*

nucleotide sequence within an autonomously-replicating nucleic acid sequence such as a DNA or RNA virus capable of replicating within the plant cell. The invention also extends to seeds from such transgenic plants. Such seeds, especially if colored, are useful as proprietary tags for plants. Any and all methods for introducing genetic material into plant cells are encompassed by the present invention.

Another aspect of the present invention contemplates the use of the extracts from transgenic plants or plant parts of transgenic plants or progeny of the transgenic plants containing all or part of the nucleic acid sequences of the present invention and, in particular, the extracts from those transgenic plants when used as a flavoring or food additive or health product or beverage or juice or coloring.

Plant parts contemplated by the present invention includes, but is not limited to flowers, fruits, nuts, roots, stems, leaves or seeds.

The extracts of the present invention may be derived from the plants or plant part in a number of different ways including chemical extraction or heat extraction or filtration or squeezing or pulverization.

The plant, plant part or extract can be utilized in any number of different ways such as for the production of a flavoring (e.g. a food essence), a food additive (e.g. a stabilizer, a colorant) a health product (e.g. an antioxidant, a tablet) a beverage (e.g. wine, spirit, tea) or a juice (e.g. fruit juice) or coloring (e.g. food coloring, fabric coloring, dye, paint).

A further aspect of the present invention is directed to recombinant forms of FMT. The recombinant forms of the enzyme will provide a source of material for research to develop, for example, more active enzymes and may be useful in developing *in vitro* systems for production of colored compounds.

Still a further aspect of the present invention contemplates the use of the genetic sequences described herein in the manufacture of a genetic construct capable of expressing an *FMT* or down-regulating an indigenous FMT enzyme in a plant.

Another aspect of the present invention is directed to a prokaryotic or eukaryotic organism carrying a genetic sequence encoding an *FMT* extrachromasomally in plasmid form.

The present invention further extends to a recombinant polypeptide comprising a sequence of amino acids substantially as set forth in SEQ ID NO:2 or SEQ ID NO:5 or SEQ ID NO:7 or SEQ ID NO:12 or SEQ ID NO:22 or SEQ ID NO:42 or SEQ ID NO:43 or an amino acid sequence having at least about 50% similarity to SEQ ID NO:2 or SEQ ID NO:5 or SEQ ID NO:7 or SEQ ID NO:12 or SEQ ID NO:22 or SEQ ID NO:42 or SEQ ID NO:43 or a derivative of said polypeptide.

A "recombinant polypeptide" means a polypeptide encoded by a nucleotide sequence introduced into a cell directly or indirectly by human intervention or into a parent or other relative or precursor of the cell. A recombinant polypeptide may also be made using cell-free, *in vitro* transcription systems. The term "recombinant polypeptide" includes an isolated polypeptide or when present is a cell or cell preparation. It may also be in a plant or parts of a plant regenerated from a cell which produces said polypeptide.

A "polypeptide" includes a peptide or protein and is encompassed by the term "enzyme".

The recombinant polypeptide may also be a fusion molecule comprising two or more heterologous amino acid sequences.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

Plant material

The *Petunia hybrida* cultivars used are presented in Table 3.

TABLE 3

Genotypes of Petunia hybrida cultivars

Plant variety	Properties	Source/Reference
V23	*An1, An2, An3, An4, An6, An8, An9, An10, ph1, Hf1, Hf2, ht1, Rt, po, Bl, Fl*	Wallroth *et al.* (*Mol. Gen. Genet. 202*: 6-15, 1986) Doodeman *et al.* (*Theor. Appl. Genet. 67*: 357-366, 1984)
R51	*An1, An2, An3, an4, An6, An8, An9, An10, An11, Ph1, hf1, hf2, Ht1, rt, Po, bl, fl*	Wallroth *et al.* (1986), *supra* Doodeman *et al.* (1984), *supra*
VR	V23 x R51 F1 Hybrid	
Br140	*An1, An2, an4, an6/An6*, Ph1, Ph2, Ph5, Hf1, Ht1, Rt, po, Mt1, mf1, mf2, Gf, fl*	INRA
Br140w	*An1, An2, an4, an6*, Ph1, Ph2, Ph5, Hf1, Ht1, Rt, po, Mt1, mf1, mf2, Gf, fl*	white flowering plants of a Br140 self
Br140p	*An1, An2, an4, an6/An6*, Ph1, Ph2, Ph5, Hf1, Ht1, Rt, po, Mt1, mf1, mf2, Gf, fl*	purple flowering plants of a Br140 self
Old Glory Blue (OGB)	F_1 Hybrid (commercial cultivar)	Ball Seed, USA
V26	*An1, An2, An3, an4, An6, An8, An9, An10, An11, Ph1, ph2, Ph5, Hf1, hf2, Ht1, Rt, po, Bl, Gf, Mt1, Mt2, mf1, mf2, Fl*	INRA
W162	*an1*	Vrije Universiteit, Amsterdam

INRA = Institut National de la Recherche Agronomique, Cedex, France

OGB petunia plants were grown in specialized growth rooms with a 14 hr day length at a light intensity of 10,000 lux and a temperature of 22 to 26°C. OGB flowers were harvested at developmental stages defined as follows:-

Stage 1: Unpigmented, closed bud (< 25 mm in length).

Stage 2: Pigmented, closed bud (25-35 mm in length).

Stage 3: Dark purple bud with emerging corolla (> 35 mm in length).

Stage 4: Dark purple opened flower pre-anther dehiscence (> 50 mm in length).

Stage 5: Fully opened flower with all anthers dehisced.

EXAMPLE 2

General Methods

In general, the methods followed were as described in Sambrook *et al.* (1989), *supra*.

E. coli transformation

The *Escherichia coli* strains used were:-

DH5α supE44, Δ(lacZYA-ArgF)U169, (ø80lacZΔM15), hsdR17(r_k^-, m_k^+), recA1, endA1, gyrA96, thi-1, relA1, deoR. (Hanahan, *J. Mol. Biol. 166*: 557, 1983 and *Bethesda Res. Lab. Focus. 8(2)*: 9, 1986).

XL1-Blue supE44, hsdR17(r_k^-, m_k^+), recA1, endA1, gyrA96, thi-1, relA1, lac^-,[F'proAB, $lacI^q$, lacZΔM15, Tn10(tet^R)] (Bullock *et al.*, *Biotechniques 5*: 376, 1987).

PLK-F' recA, hsdR17(r_k^-, m_k^+), $mcrA^-$, $mcrB^-$, lac^-, supE44, galK2, galT22, metB1, [F' proAB, $lacI^q$, lacZΔM15, Tn10(tet^R)] (Stratagene).

M15 *E. coli* is derived from *E. coli* K12 and has the phenotype Nal^s, Str^s, Rif^s, Thi^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+

The cloning vectors pBluescript, pBluescribe and PCRscript were obtained from Stratagene. pCR 2.1 was obtained from Invitrogen.

The bacterial expression vectors pQE-30 and pREP4 were obtained from QIAGEN.

Transformation of the *E. coli* strains was performed according to the method of Inoue *et al.*, (*Gene 96*: 23-28, 1990).

DNA ligations

DNA ligations were carried out using the Amersham Ligation Kit according to procedures recommended by the manufacturer.

Isolation and purification of fragments

Fragments were generally isolated on a 1% w/v agarose gel and purified using the QIAEX II Gel Extraction kit (QIAGEN).

Reparation of overhanging ends after restriction digestion

Overhanging 5' ends were repaired using DNA polymerase (Klenow fragment) according to standard protocols (Sambrook *et al.*, 1989, *supra*). Overhanging 3' ends were repaired using T4 DNA polymerase according to standard protocols (Sambrook *et al.*, 1989, *supra*).

Removal of phosphoryl groups from nucleic acids

Shrimp alkaline phosphatase (SAP) (USB) was typically used to remove phosphoryl groups from cloning vectors to prevent re-circularization according to the manufacturer's recommendations.

^{32}P-Labelling of DNA Probes

DNA fragments (50 to 100 ng) were radioactively labelled with 50 μCi of [α-^{32}P]-dCTP using a Gigaprime kit (Geneworks). Unincorporated [α-^{32}P]-dCTP was removed by chromatography on a Sephadex G-50 (Fine) column.

Plasmid Isolation

Helper phage R408 (Stratagene) was used to excise pBluescript phagemids containing petunia cDNA inserts from the amplified λZAP cDNA libraries using methods described by the manufacturer. *E. coli* XL1-Blue were transfected with the phagemid mixture and the colonies were plated out on LB plates (Sambrook *et al.*, 1989, *supra*) containing 100 μg/mL ampicillin. Single colonies were analyzed for cDNA inserts by growing in LB broth (Sambrook *et al.*, 1989, *supra*) with ampicillin (100 μg/mL) (or other appropriate antibiotic) and isolating the plasmid using the alkali-lysis procedure (Sambrook *et al.*, 1989, *supra*) or using the WizardPlus SV minipreps DNA purification system (PROMEGA). Once the presence of a cDNA insert had been determined, larger amounts of plasmid DNA were prepared from 50 mL overnight cultures using a QIAfilter Plasmid midi kit (QIAGEN).

DNA Sequence Analysis

DNA sequencing was performed using the ABI PRISM (registered trademark) BigDye (trademark) Primer Cycle Sequencing Kits from Applied Biosystems. The protocols supplied by the manufacturer were followed. The cycle sequencing reactions were performed using a Perkin Elmer PCR machine (GeneAmp PCR System 9600). Sequencing runs were performed by AGRF (Australian Genome Research Facility) at WEHI (The Walter and Eliza Hall Institute of Medical Research) in Melbourne, Australia.

Homology searches against Genbank, SWISS-PROT and EMBL databases were performed using the FASTA and TFASTA programs (Pearson and Lipman, *Proc. Natl. Acad. Sci. USA 85*: 2444-2448, 1988) or BLAST programs (Altschul *et al.*, *J. Mol. Biol. 215:* 403-410, 1990). Percentage sequence similarities were obtained using the LFASTA program (Pearson and Lipman, 1988, *supra*). In all cases, ktup values of 6 for nucleotide sequence comparisons and two for amino acid sequence comparisons were used, unless otherwise specified.

Multiple sequence alignments and dendogram plots were produced using ClustalW (Thompson *et al.*, *Nucl. Acids Res. 2*: 4673-4680, 1994).

EXAMPLE 3

Plant transformations

Agrobacterium tumefaciens transformations

The disarmed *Agrobacterium tumefaciens* strain used was AGL0 (Lazo *et al.*, *Bio/technology 9*: 963-967, 1991).

Plasmid DNA was introduced into the *Agrobacterium tumefaciens* strain AGL0 by adding 5 μg of plasmid DNA to 100 μL of competent AGL0 cells prepared by inoculating a 50 mL LB culture (Sambrook *et al.*, 1989, *supra*) and incubation for 16 hours with shaking at 28°C. The cells were then pelleted and resuspended in 0.5mL of 85% v/v 100mM $CaCl_2$/15% v/v glycerol. The DNA-*Agrobacterium* mixture was frozen by incubation in liquid N_2 for 2 minutes and then allowed to thaw by incubation at 37°C for 5 minutes. The DNA/bacterial mix was then placed on ice for a further 10 minutes. The cells were then mixed with 1 mL of LB (Sambrook *et al.*, 1989 *supra*) media and incubated with shaking for 16 hours at 28°C. Cells of *A. tumefaciens* carrying the plasmid were selected on LB agar plates containing appropriate antibiotics such as 50 μg/mL tetracycline or 100 μg/mL gentamycin or 30 μg/mL kanamycin. The confirmation of the plasmid in *A. tumefaciens* was done by

restriction endonuclease mapping of DNA isolated from the antibiotic-resistant transformants.

Petunia hybrida transformations

As described in Holton *et al.* (*Nature, 366*: 276-279, 1993) or Brugliera *et al.*, (*Plant J. 5*, 81-92, 1994) by any other method well known in the art.

(a) Plant Material

Leaf tissue from mature plants of *P. hybrida* cv VR was treated with 1.25% w/v sodium hypochlorite for 2 minutes and then rinsed three times in sterile water. The leaf tissue was then cut into 25 mm^2 squares and precultured on MS media (Murashige and Skoog, *Physiol. Plant 15*: 73-97, 1962) supplemented with 0.05 mg/L kinetin and 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) for 24 hours.

(b) Co-cultivation of Agrobacterium and Petunia Tissue

A. tumefaciens strain AGL0 (Lazo *et al.*, 1991, *supra*) containing the binary vector were maintained at 4°C on MG/L (Garfinkel and Nester, *J. Bacteriol. 144*:732-743, 1980) or LB agar (Sambrook *et al.*, 1989, *supra*) plates containing the appropriate antibiotic. A single colony used to inoculate an overnight liquid culture containing 1% w/v Bacto-peptone, 0.5% w/v Bacto-yeast extract and 1% w/v NaCl. A final concentration of 5 x 10^8 cells/mL was prepared the next day by dilution in liquid MS medium containing B5 vitamins (Gamborg *et al.*, *Exp. Cell Res. 50*: 151-158, 1968) and 3% w/v sucrose (BPM). The leaf discs were dipped for 2 minutes into BPM containing the transformed AGL0 as described above. The leaf discs were then blotted dry and placed on co-cultivation media for 4 days. The co-cultivation medium consisted of SH medium (Schenk and Hildebrandt, *Can. J. Bot. 50*: 199-204, 1972) supplemented with 0.05 mg/L kinetin and 1.0 mg/L 2,4-D and included a feeder layer of tobacco cell suspension spread over the co-cultivation medium with a filter paper placed on top of the tobacco cell suspension.

(c) Recovery of transgenic petunia plants

After co-cultivation, the leaf discs were transferred to MS medium supplemented with 3% w/v sucrose, 1 mg/L α-benzylaminopurine (BAP), 0.1 mg/L α-naphthalene acetic acid (NAA), 2 μg/L Chlorsulfuron (Chem Service), 350 mg/L cefotaxime and 0.3% w/v Gelrite Gellan Gum (Schweizerhall) (selection medium). Regenerating explants were transferred to fresh selection medium after 4 weeks. Adventitious shoots which survived the Chlorsulfuron selection were isolated and transferred to BPM containing 2 μg/L Chlorsulfuron (Chem Service) and 200 mg/L cefotaxime for root induction. All cultures were maintained under a 16 hr photoperiod (60 μmol. m^{-2}, s^{-1} cool white fluorescent light) at 23 ± 2°C. When roots reached 2-3 cm in length the transgenic petunia plantlets were transferred to autoclaved Debco 51410/2 potting mix in 8 cm tubes. After 4 weeks plants were transferred into 15 cm pots using the same potting mix and maintained at 23°C under a 14 hour photoperiod (300 μmol m^{-2}, s^{-1} mercury halide light).

Rosa hybrida transformations

As described in U.S. Patent No. 542,841 (PCT/US91/04412) or Robinson and Firoozabady (*Scientia Horticulturae, 55*: 83-99, 1993), Rout *et al.* (*Scientia Horticulturae, 81*: 201-238, 1999) or Marchant *et al.* (*Molecular Breeding 4*: 187-194, 1998) or by any other method well known in the art.

Cuttings of *Rosa hybrida* were generally obtained from Van Wyk and Son Flower Supply, Victoria, Australia or Keisei Roses, Japan

Color coding

The Royal Horticultural Society's Color Chart (Kew, UK) was used to provide a description of color observed. They provide an alternative means by which to describe the color phenotypes observed. The designated numbers, however, should be taken only as a guide to the perceived colors and should not be regarded as limiting the possible colors, which may be obtained.

Construct Preparations

TABLE 4

Abbreviations used in construct preparations

ABBREVIATION	DESCRIPTION
35S 5'	~0.2 kb incorporating *Bgl*II fragment containing the promoter region from the Cauliflower Mosaic Virus 35S gene (*CaMV 35S*) (Franck *et al.*, *Cell* 21: 285-294, 1980, Guilley *et al.*, *Cell*, 30: 763-773. 1982)
e35S 5'	~0.7 kb fragment incorporating an enhanced *CaMV 35S* promoter (Mitsuhashi *et al. Plant Cell Physiol. 37*: 49-59, 1996)
GUS	ß-glucuronidase coding sequence (Jefferson, *et al.*, *EMBO J.* 6: 3901-3907, 1987)
Mac	Hybrid promoter consisting of the promoter from the *mas* gene and a *CaMV 35S* enhancer region (Comai *et al.*, *Plant Mol. Biol.* 15: 373-381, 1990)
nos 5'	Promoter region from nopaline synthase gene of *A. tumefaciens* (Depicker, A. *et al.*, *J Mol. and Appl. Genetics*, 1: 561-573, 1982)
nos 3'	Terminator region from nopaline synthase gene of *A. tumefaciens* (Depicker, A. *et al.*, 1982, *supra*)
nptII	Kanamycin-resistance gene (encodes neomycin phosophotransferase which deactivates aminoglycoside antibiotics such as kanamycin, neomycin and G418)
ocs 3'	Terminator region from octopine synthase gene of *A. tumefaciens* (described in Klee *et al.*, *Bio/Technology 3:* 637-642, 1985)
petD8 3'	~0.8 kb fragment incorporating the terminator region from phospholipid transfer protein gene (D8) of *Petunia hybrida* cv. OGB (Holton, 1992, *supra*)

ABBREVIATION	DESCRIPTION
SuRB	Chlorsulfuron-resistance gene (encodes Acetolactate Synthase) with its own terminator from *Nicotiana tabacum* (Lee *et al.*, *EMBO J.* 7: 1241-1248, 1988)
BP#40 or Viola F3'5'H	*~1.7kb fragment containing F3'5'H* cDNA clone from *Viola sp.* cultivar black pansy. (Australian Provisional Patent Applications No. 2002951088 and 2002952835 entitled "Genetic Sequences and uses therefor", 2002)
PFMT	*~1.0 kb fragment incorporating the Petunia* flavonoid methyltransferase *E20* cDNA clone (*inter alia*)
TFMT	*~ 1.0 kb fragment incorporating the Torenia* flavonoid methyltransferase cDNA clone (*inter alia*)
FFMT	*~ 1.0 kb fragment incorporating the Fuchsia* flavonoid methyltransferase cDNA clone (*inter alia*)

EXAMPLE 4

Incubation of excised petals with precursors or end products

Reports in the literature suggest that of the six principally occurring anthocyanidins found in nature (Table 5), the degree of "blueness" of an individual anthocyanidin is influenced by the hydroxylation and/or methylation pattern in the anthocyanin "B" ring. However, in 0.01% HCl/MeOH (v/v) solutions delphinidin has a higher λ_{max} value than peonidin or malvidin and so appears the bluest of the six anthocyanidins.

TABLE 5

λ_{max} values (in nm) of major anthocyanidins

ANTHOCYANIDIN	λ_{max} nm*
Pelargonidin	520
Cyanidin	535
Peonidin	532
Delphinidin	546
Petunidin	543
Malvidin	542

λ_{max} nm* wavelength of maximum absorption in 0.01%HCl/MeOH (v/v)

Data reviewed by Haslam (Practical Phenolics. From structure to molecular recognition and physiological action. Cambridge University Press, UK, 1998).

Experiments were set up to determine whether the production of delphinidin or its methylated derivative, malvidin would lead to novel colors in rose petals. To determine whether rose petals contained the enzymes necessary for conversion of dihydromyricetin to delphinidin, precursor-feeding experiments with dihydromyricetin were initiated.

Petal segments of a selection of commercial cultivars of roses (Toplesse, Lambada, Medeo, Pamela, Sonia, Oceana, Mystique) were placed in solutions of 1-2 mg/mL dihydromyrectin or water only and incubated for around 16 hours in a growth room at a temperature of around 23°C. Pink/purple colors were observed near the cut edges of the petals (Table 6). TLC analysis of the anthocyanidins in the pink/purple segments revealed the production of delphinidin. These results confirmed that the anthocyanin pathway enzymes of roses were able to convert dihydromyricetin to delphinidin.

TABLE 6

Colors produced in rose petals after incubation in dihydromyricetin (the precursor of delphinidin-based pigments)

Rose cultivar	Color of rose petal	Color at cut edge after incubation in DHM
Toplesse	pink	pink/purple
Lambada	orange	pink/purple
Medeo	pale apricot	pink/purple
Pamela	white/pale pink	pink/purple
Sonia	apricot/pink	pink/purple
Oceana	cream	pink/purple
Mystique	apricot	pink/purple

DHM = dihydromyricetin

Rose petals from Toplesse and Lambada were subsequently incubated with malvidin 3, 5-diglucoside to determine the color that may be obtained if this novel anthocyanin were to be produced in rose *via* introduction of a flavonoid 3' 5' hydroxylase gene for production of delphinidin-based pigments and an flavonoid 3' 5' methyltransferase gene (or flavonoid 3' methyltransferase and flavonoid 5' methyltransferase genes) for the subsequent conversion to malvidin-based pigments.

Petal segments of roses were placed in solutions of 1-2 mg/mL malvidin 3, 5-diglucoside, 1-2 mg/mL dihydromyrectin or water only and incubated for around 16 hours in a growth room at a temperature of around 23°C. The production of colors in the purple range were observed near the cut edges of the petals upon incubation with dihydromyrectin or malvidin 3, 5-diglucoside (Table 7). However a direct comparison of the colors observed with the production of delphinidin in the rose petals to the accumulation of malvidin in the same rose background surprisingly revealed that malvidin pigments resulted in bluer colors.

TABLE 7

Colors observed in rose petals after incubation in dihydromyricetin (the precursor of delphinidin-based pigments) or in malvidin 3, 5-diglucoside

Rose cultivar	Petal color	Color at cut edge upon production of delphinidin	Color at cut edge after incubation with malvidin
Toplesse	pink	pink/purple	violet/purple
Lambada	orange	pink/purple	violet/purple

Reconstruction experiments

Reconstruction experiments with rose petal extracts and various anthocyanins were performed to predict the color that would be produced in roses upon production of delphinidin or malvidin-based pigments.

The rose cultivar Medeo generally produces cream-colored to pale apricot flowers (RHSCC 158C to 159A). HPLC analysis of the anthocyanidins and flavonols accumulating in Medeo rose petals revealed that the petals accumulate high levels of flavonols (2.32 mg/g kaempferol, 0.03 mg/g quercetin) and very low levels of anthocyanins (0.004 mg/g cyanidin, 0.004 mg/g pelargonidin). The estimated vacuolar pH of Medeo petals is around 4.6. The petal juice of Medeo roses was extracted by grinding one petal with 50 µL of water using a mortar and pestle. The petal juice was collected and mixed with 10-20 µL of 1-2 mg/g delphinidin 3-glucoside, delphinidin 3,5-diglucoside and malvidin 3, 5-diglucoside. The colors observed were described according to the Royal Horticultural Society Color Charts (RHSCC) (The Royal Horticultural Society, London) (Table 8).

TABLE 8

Colors observed upon addition of delphinidin 3-glucoside, delphinidin 3, 5-diglucoside or malvidin 3, 5-diglucoside to petal juice extracted from Medeo rose petals

ANTHOCYANIN	pH	RHSCC#	COLOR
D3G	4.9	74A	red-purple
D35G	4.9	88A	violet
D35G	4.6	88A	violet
M35G	4.9	90A	violet-blue
M35G	4.6	88A/90A	violet-blue

D3G = delphinidin 3-glucoside,

D35G = delphinidin 3, 5-diglucoside
M35G = malvidin 3, 5-diglucoside

Based on the λ_{max} value (Table 5), it was assumed that the production of delphinidin pigments in rose petals would result in a bluer color than the production of malvidin pigments. However, from the feeding and reconstruction experiments detailed above it is clear that the production of malvidin-based pigments in rose petals will lead to bluer colors than that of delphinidin-based pigments.

EXAMPLE 5

Isolation of a partial S-adenosyl-L-methionine: flavonoid methyltransferase (FMT) cDNA clone from Petunia hybrida

Construction and screening of a P. hybrida cv. V26 petal cDNA library

A cDNA library was constructed based on mRNA from corolla limb tissue of the line V26 (*An1*$^+$) (Kroon *et al.*, *Plant J 5*: 69-80, 1994). Around 30,000 pfu of the V26 floral cDNA library were plated at a density of 800 pfu per 90 mm plate. Duplicate lifts of these were taken onto Hybond-N membranes (Amersham) and treated as recommended by the manufacturer. The filters were hybridized with first strand cDNA from an *An1*$^+$ (V26) and an *an1*$^-$ line (W162). Hybridization conditions included a prehybridization step in 50% v/v formamide, 5 x SSPE, 5 x Denhardt's, 0.1% w/v SDS, 100 µg/mL herring sperm DNA at 42°C for 3 hours. For hybridization 1.0 x 10^8 cpm ^{32}P-labeled first strand cDNA and 100 µg of poly (A) were added and incubation was continued for 16-48 hours at 42°C. The filters were washed in 1 x SSC/0.1% w/v SDS at 60°C for 30 minutes and then exposed to Kodak XAR film for 3 to 4 days. Two hundred and seventy plaque forming units (pfu) out of 30,000 showed substantially stronger hybridization to the *An1*$^+$ cDNA probe than to the *an1*$^-$ cDNA probe. Of these, 35 which did not hybridize to previously cloned pigmentation genes (*chs*, *chi* and *dfr*) were purified to homogeneity. Pairwise cross-hybridizations demonstrated that these 35 clones represented 7 distinct classes of genes-*difA*, *difC*, *difE*, *difF*, *difG*, *difH* and *difI*. The *difG gene* has subsequently been shown to represent the *Rt*

gene of *Petunia hybrida* (Kroon *et al.*, 1994, *supra*). The expression profiles of the remaining 6 classes were shown to display a spatial, temporal and genetic control similar to that of *difG* (Kroon *et al.*, 1994, *supra*).

The *difC* clone was subsequently shown to represent the anthocyanidin 3-rutinoside acyltransferase (AR-AT) gene of *Petunia hybrida* (International Application No. PCT/AU01/00358; International Publication No. WO 01/72984).

The *difE* clone was shown to be around 1kb and the plasmid was assigned the designation pCGP1903 (Figure 2). The complete sequence of the *difE* cDNA clone (SEQ ID NO:1) (contained in pCGP1903) was determined by compilation of sequence from different pUC18 subclones obtained using standard procedures for the generation of randomly-overlapping clones (Sambrook *et al.*, 1989, *supra*). Blast searches against sequences in the GenBank database revealed similarities to caffeoyl-CoA *O*-methyltransferase mRNAs. (e.g. 84% identity over a 92 bp span of *Mesembryanthemum crystallinum* caffeoyl-CoA *O*-methyltransferase (AF053553)).

RFLP analysis indicated that the *difE* clone was linked to the *Hf2* and *Po* loci (5 cross overs out of 33 plants with the *Po* locus and 8 cross overs with *Hf2* out of 34 plants) on chromosome V and so was a candidate for the *Mt2 or Mf2* gene. RNA gel blots were subsequently performed on various *Mf* and *Mt* mutants and it was shown that four-double mutants (*mf1-*, *mf2-*, *mt1-*, *mt2-*) lacked transcripts hybridising to *difE*, while lines dominant for one or more of these loci did contain *difE* transcripts. This suggested that the *difE* clone encoded a flavonoid methyltransferase and that the different *FMT* transcripts cross-hybridise. The *difE* clone was selected for further analysis.

EXAMPLE 6

Isolation of a full-length FMT cDNA clone from Petunia hybrida cv Old Glory Blue (OGB)

Construction of OGB petal cDNA library

Total RNA was isolated from the petal tissue of *P. hybrida* cv Old Glory Blue (OGB) stage 3 to 4 flowers using the method of Turpen and Griffith (*BioTechniques 4:* 11-15, 1986). Poly(A)$^+$ RNA was selected from the total RNA by three cycles of oligo-dT cellulose chromatography (Aviv and Leder, *Proc. Natl. Acad. Sci. USA 69*: 1408, 1972).

Two micrograms of poly(A)$^+$ RNA were reverse transcribed in a 20 µL reaction volume containing 1 x Superscript (trademark) reaction buffer, 10 mM dithiothreitol, 500 µM dATP, 500 µM dGTP, 500 µM dTTP, 500 µM 5-methyl-dCTP, 0.75 µg oligonucleotide (5' GAGAGAGAGAGAGAGAGATCTCGAGTTTTTTTTTTTTTTTT) [SEQ ID NO:3] and 2 µL Superscript (trademark) reverse transcriptase (BRL). The reaction mix was incubated at 37°C for 50 minutes, 44°C for 10 minutes and then placed on ice.

A second strand reaction mix (140 µL) was added to the first strand reaction mix. The second strand reaction mix consisted of 21 mM Tris-HCl, 104 mM KCl, 5.3 mM $MgCl_2$, 171 µM β-NAD, 11.4 mM $(NH_4)_2SO_4$, 214 µM dATP, 642 µM dCTP, 214 µM dGTP, 214 µM dTTP, 4 mM DTT, 10 µCi ^{32}P-dCTP (3000 Ci/mMole), 15 units *E. coli* DNA ligase, 40 units *E. coli* DNA polymerase I (Boehringer) and 0.8 units RNAse H. The final mixture was incubated for 150 minutes at 16°C. To make the double-stranded cDNA blunt-ended, 10 units T4 DNA polymerase was added, and the reaction continued for a further 15 minutes at 16°C. The reaction was stopped and the cDNA purified by phenol/chloroform extraction, followed by chloroform extraction and ethanol precipitation.

*Eco*RI adaptors (Promega) were ligated with the cDNA and then kinased using conditions recommended by the manufacturer. The enzymes were denatured by heat (70°C, 20 minutes) and the DNA was purified by phenol/chloroform extraction and ethanol

precipitation. The cDNA was digested with 50 units of *Xho*I restriction endonuclease (Boehringer Mannheim) in a reaction volume of 100 µL, using conditions recommended by the manufacturer. The enzyme was heat killed (70°C, 20 minutes) and the mixture passed through an S400 spin column (Pharmacia) which had been equilibrated in STE buffer (Sambrook *et al.*, 1989, *supra*). The eluate was phenol/chloroform extracted and ethanol precipitated. After microcentrifugation at 4°C for 30 minutes the resulting cDNA pellet was rinsed with 70% v/v ethanol, air dried and resuspended in 10 µL of TE buffer (1 mM Tris-HCl (pH 7.5), 1 mM EDTA).

A 2.5 µL aliquot of the resuspended cDNA mixture was ligated with 1 µg λZAPII *Eco*RI/*Xho*I/CIAP (calf intestinal alkaline phosphatase) treated vector (Stratagene) in 5 µL of reaction buffer consisting of 50 mM Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and 2 units of T4 DNA ligase. The reaction was carried out at 4°C for 4 days.

After a subsequent incubation at room temperature for two hours, the ligation reaction mixture was packaged using the Packagene system (Promega). The total number of recombinants was 1 x 10^6 pfu.

After transfecting PLK-F' cells, the packaged λZAPII/cDNA was plated at 50,000 pfu per 15 cm diameter petri plate. The plates were incubated at 37°C for eight hours, and the phage were eluted in 100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl pH 8.0, 0.01% gelatin (Phage Storage Buffer (PSB)). Chloroform was added and the phage stored at 4°C as an amplified library.

40,000 pfu of the amplified library were plated onto NZY plates (Sambrook *et al.*, 1989, *supra*) at a density of 20,000 pfu per 15 cm plate after transfecting XL1-Blue MRF' cells, and incubated at 37°C for 8 hours. After a subsequent incubation at 4°C overnight, duplicate lifts were taken onto Colony/Plaque Screen (trademark) filters (DuPont) which were then treated as recommended by the manufacturer.

Screening of OGB Library

Prior to hybridization, the duplicate plaque lifts were washed in pre-washing solution (50 mM Tris-HCl pH 7.5, 1 M NaCl, 1 mM EDTA, 0.1% w/v sarcosine) at 65°C for 30 minutes; stripped in 0.4 M sodium hydroxide at 65°C for 30 minutes; then washed in a solution of 0.2 M Tris-HCl pH 8.0, 0.1 x SSC, 0.1% w/v SDS at 65°C for 30 minutes and finally rinsed in 2 x SSC, 1.0% w/v SDS.

The duplicate lifts from the OGB petal cDNA library were screened with ^{32}P-labelled fragments of an *Eco*RI/*Xho*I *difE* fragment from pCGP1903 (Figure 2).

Hybridization conditions included a prehybridization step in 50% v/v formamide, 1 M NaCl, 10% w/v dextran sulphate, 1% w/v SDS at 42°C for at least 1 hour. The ^{32}P-labeled fragments (at 1 x 10^6 cpm/mL) were then added to the hybridization solution and hybridization was continued at 42°C for a further 16 hours. The filters were then washed in 2 x SSC, 1% w/v SDS at 42°C for 2 x 30 minutes followed by a wash in 0.2 x SSC, 1% w/v SDS at 65°C for 30 minutes and exposed to Kodak XAR film with an intensifying screen at -70°C for 4 hours.

Forty-five hybridizing plaques (designated as *E1* to *E45*) were picked into PSB. These were rescreened to isolate pure clones, using the hybridization conditions as described for the initial screening of the cDNA library. The plasmids contained in the λZAP bacteriophage vector were rescued and sequence data was generated from the 3' and 5' ends of the cDNA inserts. Of these *E20* and *E33* represented the longest cDNA clones (~1.0kb and 0.9kb, respectively) and the plasmids were designated pCGP1907 and pCGP1908 (Figures 3 and 4, respectively).

The complete nucleotide sequence of the *E20* and *E33* cDNA clones (SEQ ID NOs:4 and 6) (contained in pCGP1907 and pCGP1908, respectively) was determined by compilation of sequence generated using commercially available M13 reverse and M13 -21 primers along with a specific *Petunia* MT primer 1903F (5' CTT GCT TTG CCA GAA GAT GG

3') [SEQ ID NO:8]. The *E20* cDNA clone was 888 bp in length and contained a putative open reading frame of 789 bases which encoded a putative polypeptide of 263 amino acids (SEQ ID NO:5). The *E20* sequence was identical to the *difE* sequence over 822 bp with the *E20* cDNA clone having an extra 27bp of 5' untranslated sequence and a reduction of 96 bp of 3' untranslated sequence as compared to the *difE* sequence.

The *E33* sequence was 1076 bp in length and contained an in-frame stop codon at position 469 (SEQ ID NO:6). The *E20* sequence shared 82% identity over 797 bp with the *E33* sequence at the nucleotide level. An alignment of the *E33* nucleotide sequence with that of the *E20* sequence revealed an apparent 2 nucleotide ("CT") deletion in the *E33* sequence resulting in an in-frame stop codon. It may be that the *E33* clone in the OGB cultivar was derived from a mutated gene. In order to examine the presumed deduced amino acid sequence of the non-mutated gene represented by the *E33* clone, 2 nucleotides ("CT") were added to the *E33* sequence to produce the *E33*-corrected nucleotide sequence (SEQ ID NO:26). The deduced amino acid sequence is represented by SEQ ID NO:7. The E33-corrected amino acid sequence shared an 82% identity with the E20 sequence over a 243 amino acid overlap.

A comparison of the translated nucleotide sequence of *E20* to sequences in the GenBank database revealed similarity to various caffeoyl-CoA 3-*O*-methyltransferases. For example, 60% identity over 227 amino acids with Caffeoyl-CoA 3-*O*-methyltransferase from *Populus kitakamiensis* (Genbank accession number AB000408) and 53% identity over 238 amino acids of a trans-caffeoyl-CoA 3-*O*-methylransferase (CCOFMT) (CCOAOMT) from *Petroselinum crispum* (Genbank accession number A40975).

EXAMPLE 7

Methyltransferase activity of the Petunia FMT (E20) cDNA clone expressed in E. coli

To confirm whether the *Petunia E20* cDNA clone encoded a functional FMT it was expressed in an *E. coli* expression system and assayed for FMT activity.

Cloning of E20 into pQE30 E. coli expression vector (Construction of pCGP3086)

In order to clone the *Petunia E20* clone (*PFMT*) into an *E. coli* expression vector, pQE30 (QIAGEN), a *Bam*HI restriction endonuclease site was required at the translation initiating ATG and a *Pst*I restriction endonuclease site was required immediately 3' to the putative stop codon.

The oligonucleotides 1907*Bam*HI F [SEQ ID NO:9] and 1907*Pst*I R (SEQ ID NO:10) (Table 9) were used as primers with pCGP1907 as template to amplify the *Petunia FMT* clone (*E20*) with a *Bam*HI restriction endonuclease recognition site in place of the initiating AUG and a *Pst*I restriction endonuclease recognition site just after the putative stop codon. PCR conditions included 5 µL 10 x PfuTurbo DNA Polymerase buffer (Stratagene), 2 µL 10 mM dNTPs, 2 µL 20µ/µL 1907*Bam*HI F [SEQ ID NO:9], 2 µL 20 µ/µL 1907*Pst*I R [SEQ ID NO: 10], 1 µL 1 µg/µL pCGP1907 template, 37 µL pure water and 1 µL PfuTurbo DNA Polymerase (Stratagene). The PCR was incubated at 95°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute and then a final incubation at 72°C for 10 minutes with subsequent storage at 4°C.

TABLE 9

Oligonucleotides used in the cloning of the E20 cDNA clone into pQE30 bacterial expression vector

SEQ ID NO:	NAME	SEQUENCE

9	1907*Bam*HI F	GCAT GGA TCC ACA GGC AAA ACC GCC CAC CCT G
10	1907*Pst*I R	GCAT CTG CAG CTA GGA GAG ACG CCT GCA AAG

The resulting PCR products were electrophoresed through a 1% w/v agarose gel and a 0.72 kb band was isolated and purified using a QIAEX II Gel Extraction kit (QIAGEN) according to manufacturer's recommendation. The isolated products were then digested the restriction endonuclease *Pst*I. The digestion product was purified using a QIAquick PCR purification kit (QIAGEN) and then digested with the restriction endonuclease *Bam*HI. The *Bam*HI/*Pst*I digested products were finally purified using a QIAquick PCR purification kit (QIAGEN) and subsequently ligated with the *Bam*HI/*Pst*I ends of the pQE30 vector (QIAGEN) using a DNA Ligation Kit (Amersham) according to the manufacturer's recommendations. Transformants were analyzed for the presence of the specific 0.72 kb insert using *BamHI*/*Pst*I restriction endonuclease digests. The sequence of the insert was confirmed by sequence analysis using a pQE Sequencing-Primer Set (QIAGEN). The resulting plasmid was designated pCGP3086 (mut-*E20* in pQE30) (Figure 5).

As a consequence of using the 1907*Bam*HI F [SEQ ID NO: 9] and 1907*Pst*R [SEQ ID NO: 10] oligonucleotides as primers in the PCR and of the subsequent cloning of the product into pQE30, the sequence of the *Petunia E20* clone was altered around the putative initiating methionine of the encoded polypeptide. As a consequence the expected amino acids around the putative initiating methionine were changed from "M T G K T A H P" to "M R G S H H H H H H G S T G K T A H P".

According to the manufacturer, "the 6 x His-tag is much smaller than most other affinity tags and is uncharged at physiological pH. It rarely alters or contributes to protein immunogenicity, rarely interferes with protein structure or function, does not interfere with secretion, does not require removal by protease cleavage, and is compatible with denaturing buffer systems". (QIAGEN website, http://www.qiagen.com).

For analysis of methyltransferase activity of the *E20* clone, pCGP3086 was subsequently introduced into *E. coli* M15 ($pREP_4$) (QIAGEN) cells according to the method of Inoue *et al.*, 1990, *supra*

10 mL of LB containing ampicillin at 100 μg/mL (LB/Amp100) was inoculated with a single colony of pCGP3086 in M15/$pREP_4$cells and incubated at 37°C with shaking for 16 hours. One millilitre of this culture was then used to inoculate 25 mL LB/Amp100. The culture was incubated at 37°C with shaking for around 2 hours until the Absorbance at 600 nm (A_{600}) was between 0.5 to 0.7. IPTG (iso-propyl-ß-D-thiogalactoside) was then added to a final concentration of 1 mM and the culture was further incubated at 37°C with shaking with 1.5 mL aliquots being removed at 0, 1, 2 and 5 hours after addition of IPTG.

The cells contained in each aliquot were subsequently pelleted by centrifugation and then resuspended in 50 μL 8 M urea denaturing buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01M Tris-HCl, pH8). The lysates were centrifuged at 14,000 rpm for 10 minutes at room temperature to pellet cell debris. The crude protein extracts were denatured by boiling in 10% glycerol, 3% w/v sodium dodecyl sulphate (SDS), 3% β-mercaptoethanol (BME) and 0.025% bromophenol blue and then electrophoresed through precast SDS PAGE gels (12% resolving, 4% stacking gel) (Ready Gels, BIORAD) in a running buffer made up of 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% w/v SDS at 120V for 80 min. Standards included prestained Low Range markers (BIORAD) which contained standard protein samples of 116 kDa, 80 kDa, 51.8 kDa and 34.7 kDa.

Proteins were visualized by staining with Coomassie Brilliant Blue (CBB) (0.25% w/v CBB, 45% v/v methanol. 10% v/v acetic acid). A strongly staining band of the size expected for a His-Tag fusion of *Petunia* FMT (E20) protein was detected at 27.5 kDa. Proteins on a duplicate SDS-PAGE gel to that described above were also electro-transferred to Immun-blot PVDF membrane (BIORAD) at 4°C in a buffer of 25 mM Tris-HCl pH 8.3, 20% methanol and 192 mM glycine at 100V for 60 min. The presence of the His-tag fused to the specific protein encoded by the *E20* cDNA clone in pCGP3086 was confirmed by detection with a Ni-NTA-AP conjugate (QIAGEN) according to the

manufacturer's instructions. A strongly staining protein band estimated to be 27.5 kDa was detected confirming the presence and high level expression of the recombinant E20 protein. No bands were visible in a pQE30 control under these detection conditions.

Preparation of crude protein extracts

10 mLs of LB containing ampicillin at 100 μg/mL and kanamycin at 25 μg/mL (LB/Amp100 + Kan25) were inoculated with a single colony of pCGP3086 or pQE30 in M15 ($pREP_4$) cells. The culture was incubated at 30°C with shaking for 16 hours. 2.5 mL of this culture was then added to 25 mL fresh LB/Amp100+Kan25 and the freshly inoculated culture was incubated at 30°C with shaking until an A_{600} of 0.5 to 0.7 was reached. IPTG was then added to a final concentration of 1mM and the culture was further incubated at 30°C with shaking for 8 hours. The cells were pelleted by centrifugation at 3500 rpm for 10 minutes at 4°C. The pellet was resuspended in 1 mL of 0.1 M NaPi, pH 7.5, 4 mM $MgCl_2$. Freshly prepared lysozyme was then added to a final concentration of 1mg/mL and the mixture was incubated on ice for 30 minutes. The mixture was then sonicated for two bursts of 10 seconds at output 2-3 and then incubated on ice for 30 minutes. The cell debris was pelleted by centrifugation at 14,000 rpm for 20 minutes at 4°C. The supernatant was passed through a NAP-10 column (Pharmacia) and 1.5 mL of the sample collected in 0.1 M NaPi, pH 7.5, 4 mM $MgCl_2$.

Methyltransferase activity

The enzyme activity of the *Petunia E20* clone contained in pCGP3086 was initially assessed using the substrates delphinidin 3-glucoside and delphinidin 3-rutinoside under assay conditions as described in Jonsson *et al.* (1983), *supra*.

Methyltransferase assays were set up according to Table 10 in a total reaction volume of 50 μL.

TABLE 10: Composition of methyltransferase assays using crude homogenates from bacterial cultures containing the plasmids pCGP3086 (*E20*) or pQE30 (control).

#	Plasmid	Crude homogenate (µL)	3 mg/mL D3R (µL)	3 mg/mL D3G (µL)	^{14}C-SAM (µL)	Buffer (µL)
1	pQE30	20	5	0	5	20
2	pQE30	20	-	5	5	20
3	pQE30	20	5	0	0	25
4	pQE30	20	0	5	0	25
5	pQE30	20	0	0	5	25
6	none	0	5	0	5	40
7	none	0	0	5	5	40
8	pCGP3086	20	5	0	5	20
9	pCGP3086	20	-	5	5	20
10	pCGP3086	20	5	0	0	25
11	pCGP3086	20	0	5	0	25

# = tube number,
D3G = delphinidin 3-glucoside,
D3R = delphinidin 3-rutinoside,
^{14}C-SAM = 0.6 M ^{14}C-SAM (13 µCi/µmol) (Amersham Pharmacia),
Buffer = 0.1 M NaPi, pH 7.5, 4 mM $MgCl_2$

The assay reactions were incubated at 30°C for 30 minutes. Fifty microlitres of a chloroform mix ($CHCl_3$:methanol/1% HCl, 2:1) was added and the mixture was then vortexed to stop the reactions. The phases were separated by centrifugation at 13,000 rpm for 5 minutes and 50 µL of the upper phase was transferred into a clean tube and the contents subsequently hydrolysed by the addition of 12.5 µL of 10M HCl. The tube was then placed in a boiling waterbath for 30 minutes and the contents subsequently desiccated under vacuum. The residue was resuspended in 2-3 µL of methanol/1% HCl and spotted onto a TLC plate alongside standard samples of petunidin, malvidin and delphinidin. The

anthocyanidins were separated in a Forestal system (HOAc: water: HCl; 30: 10: 3) (Markham, *Techniques of flavonoid identification.*, Academic Press, London, 1982) and the TLC was exposed to an autoradiographic film (Kodak) for 16 hours at -70°C.

TABLE 11

Results of methyltransferase assays of extracts of E. coli containing pCGP3086 or pQE30 control vector using delphinidin 3-glucoside or delphinidin 3-rutinoside as substrate and ^{14}C-SAM as methyl donor

#	Plasmid	Crude homogenate	D3R	D3G	^{14}C-SAM	Petunidin	Malvidin
1	pQE30	+	+	-	+	no	no
2	pQE30	+	-	+	+	no	no
3	pQE30	+	+	-	-	no	no
4	pQE30	+	-	+	-	no	no
5	pQE30	+	-	-	+	no	no
6	none	-	+	-	+	no	no
7	none	-	-	+	+	no	no
8	pCGP3086	+	+	-	+	yes	yes
9	pCGP3086	+	-	+	+	yes	yes
10	pCGP3086	+	+	-	-	no	no
11	pCGP3086	+	-	+	-	no	no

#	=	tube number,
D3G	=	delphinidin 3-glucoside,
D3R	=	delphinidin 3-rutinoside,
^{14}C-SAM	=	^{14}C-labelled *S*-adenosyl-L-methionine (Amersham Biosciences),
+	=	present in reaction mix
-	=	absent from reaction mix
yes	=	detection of product on TLC,
no	=	no reaction observed as determined by absence of product.

Petunidin and malvidin, the methylated derivatives of delphinidin, were detected in assay reactions using crude homogenates from pCGP3086 cells along with the substrates D3R or D3G (Tubes 8 and 9, Table 11). There was no detectable production of petunidin and malvidin in assay reactions using crude homogenates from pQE30 cells (Tubes 1 to 5, Table 11) or having no crude homogenates added (Tubes 6 and 7, Table 11) or in assay reactions without the addition of ^{14}C-SAM (Tubes 10 and 11, Table 11). The results obtained with expression of the *E20* cDNA clone in an *E. coli* expression system provide further evidence to suggest that the *E20* cDNA clone from *Petunia* codes for an FMT that is able to methylate delphinidin 3-glucoside and delphinidin 3-rutinoside using SAM as a methyl donor to produce the 3'-methylated derivative, petunidin and the 3' 5'-methylated derivative, malvidin.

EXAMPLE 8

Antisense expression of FMT in plants

The *Petunia FMT* clones (*E20* and *E33*) were each cloned in an antisense orientation behind a *Mac* promoter (Comai *et al.*, 1990, *supra*) and introduced into purple flowered VR petunia hybrid line.

Construction of pCGP40

Plasmid pCGP40 was constructed by removing the GUS gene (Jefferson *et al.*, *EMBO J. 6(13)*: 3901-3907, 1987) as a *Bam*HI-*Sac*I restriction endonuclease fragment from pCGN7334 and replacing it with the *Bam*Hl-*Sac*I restriction endonuclease fragment from pBluescribe M13$^-$ that includes the multi-cloning site. Plasmid pCGN7334, obtained from Calgene Inc. (CA, USA), was constructed by inserting the fragment containing the *Mac: GUS: mas 3'* gene fusion into the *Xho*I restriction endonuclease site of pCGN7329 (Comai *et al., Plant Molecular Biology 15:* 373-381, 1990).

Construction of pCGP1910 and pCGP1911

Plasmids pCGP1910 and pCGP1911 were constructed by cloning the respective cDNA inserts from pCGP1907 and pCGP1908 (Figure 3 and 4) in an antisense orientation behind the *Mac* promoter (Comai *et al.*, 1990, *supra*) of pCGP40. The *GUS* coding region in pCGP40 was removed by digestion with *SacI/Asp*718 restriction endonucleases. The vector containing the Mac promoter and mas terminator was purified using GeneClean Kit (Bresatec) and ligated with *Sac*I/Asp718 restriction endonuclease ends of the *Petunia E20 and E33* cDNA fragments released from pCGP1907 and pCGP1908 respectively. Correct insertion of the *E20 and E33* inserts in pCGP1910 and pCGP1911 was established by *SacI/Asp*718 restriction endonuclease analysis of DNA isolated from chloramphenicol-resistant transformants.

Plasmids pCGP1918 (Figure 6) and pCGP1919 (Figure 7) were constructed by cloning the respective *Mac: Petunia E20: mas 3'* and the *Mac: Petunia E33: mas 3'* expression cassettes from the plasmids pCGP1910 and pCGP1911 into the Ti binary vector pWTT2132 (DNAP). The *Petunia E20* and *E33* chimaeric genes were isolated from pCGP1910 and pCGP1911 upon restriction endonuclease digestion of the plasmid with *Bgl*II and the resulting 5' overhang was repaired using the Klenow fragment of DNA polymerase I. The *Petunia E20* and *E33* chimaeric genes were purified using a Bresaclean Kit (Bresatec) and then ligated with dephosphorylated *Sma*I ends of the binary vector pWTT2132. Correct ligation of the fragments was established by restriction endonuclease digestion of plasmid DNA isolated from tetracycline resistant *E. coli* transformants. The resulting plasmids were designated pCGP1918 (Figure 6) and pCGP1919 (Figure 7), respectively.

Antisense suppression of FMT activity in P. hybrida

The plasmids pCGP1918 (Figure 6) and pCGP1919 (Figure 7) were each introduced into the *Agrobacterium tumefaciens* strain AGL0 separately. The T-DNA contained in the

plasmids pCGP1918 (Figure 6) and in pCGP1919 (Figure 7) were introduced in separate experiments into *P. hybrida* cv. VR *via Agrobacterium*-mediated transformation.

Transgenic analysis of pCGP1918/VR and pCGP1919/VR petunia plants

Independent transgenic plants were produced and grown to flowering. A selection of plants produced flowers with dark pink colors, which differed to the purple colored VR control. A selection of flower colors observed is shown in Table 12. The pigments accumulating in the flowers of the transgenic plants were analyzed by HPLC (Table 13).

TABLE 12

Petal colors of VR, 1918/VR and 1919/VR transgenic flowers

ACCESSION NUMBER	CULTIVAR/ CONSTRUCT	RHSCC CODE	PETAL COLOR
9339	VR/1918	64C, 67A	dark pink
9724	VR/1918	64B, 67A	dark pink
10161	VR/1918	78A	purple
10167	VR/1918	78A	purple
10169	VR/1918	78A	purple
10171	VR/1918	78A	purple
9349	VR/1919	78A	purple
9463	VR/1919	78A	purple
10177	VR/1919	74A	red/purple
10178	VR/1919	78A	purple
10183	VR/1919	78A	purple
12704	VR control	78A	purple

RHSCC = Royal Horticultural Society Color Chart (Kew, UK).

Extraction of anthocyanidins

Prior to HPLC analysis, the anthocyanin and flavonol molecules present in petal and stamen extracts were acid hydrolyzed to remove glycosyl moieties from the anthocyanidin or flavonol core. Anthocyanidin and flavonol standards were used to help identify the compounds present in the floral extracts.

Anthocyanidins in the reaction mixture were analysed by HPLC *via* gradient elution using gradient conditions of 50%B to 60%B over 10 minutes, then 60% B for 10 minutes and finally 60% B to 100% B over 5 minutes where solvent A consisted of TFA: H_2O (5:995) and solvent B consisted of acetonitrile: TFA: H_2O (500:5:495). An Asahi Pac ODP-50 cartridge column (250 mm x 4.6 mm ID) was used for the reversed phase chromatographic separations. The flow rate was 1 mL/min and the temperature was 40°C. The detection of the anthocyanidin compounds was carried out using a Shimadzu SPD-M6A three dimensional detector at 400-650 nm.

The anthocyanidin peaks were identified by reference to known standards, *viz* delphinidin, petunidin, malvidin, cyanidin and peonidin

TABLE 13

Percentage levels of anthocyanidins detected in the petals of VR/1918 and VR/1919 transgenics by HPLC analysis

Acc#	pCGP#	Color	Anthocyanidin (%)				
			Del	Cya	Pet	Peo	Mal
9724	**1918**	**dark pink**	**51.7**	**6.0**	**34.5**	**0.0**	**7.8**
10161	1918	purple	1.2	0.6	0.6	0.3	97.3
10167	1918	purple	0.6	0.2	4.7	0.3	94.2
10169	1918	purple	0.4	0.1	4.7	0.3	94.4
10171	1918	purple	0.5	0.1	5.4	0.3	93.7
9349	1919	purple	0.6	0.0	5.6	0.2	93.6

9463	1919	purple	0.8	0.1	7.9	0.3	90.9
10177	**1919**	**red-purple**	**36.8**	**0.0**	**38.7**	**0.0**	**24.5**
10178	1919	purple	1.2	0.0	14.5	0.2	84.1
10183	1919	purple	0.5	0.0	4.4	0.3	94.8
12704	**VR control**	**purple**	**0.3**	**0.0**	**3.8**	**15.7**	**80.1**

Acc# = Accession number of plant,

pCGP# = Plasmid number,

Del = Delphinidin, expressed as a percentage of total anthocyanidins detected,

Cya = Cyanidin, expressed as a percentage of total anthocyanidins detected,

Pet = Petunidin, expressed as a percentage of total anthocyanidins detected,

Peo = Peonidin, expressed as a percentage of total anthocyanidins detected,

Mal = Malvidin, expressed as a percentage of total anthocyanidins detected

Antisense expression of *Petunia E20* (in pCGP1918) and *E33* (in pCGP1919) led to a change in flower color from purple to dark pink or red-purple with a concomitant change in the anthocyanin composition. In general, VR control petunia flowers predominantly accumulate malvidin (the 3', 5' methylated derivative of delphinidin) (around 80% of total anthocyanidin) (Table 13). The transgenic line 9724 containing the antisense *Petunia E20* gene produced flowers with a dark pink color with the predominant anthocyanin being delphinidin suggesting that the expression of the antisense *E20* gene has impacted upon a 3' 5' methyltransferase activity. The transgenic line 10177 containing the antisense *Petunia E33* gene produced flowers with a red-purple color with the predominant anthocyanins being delphinidin and petunidin suggesting that the expression of the antisense *E33* gene has also impacted upon a 3' 5' methyltransferase activity.

EXAMPLE 9

Isolation of FMT cDNA clone from Torenia

Preparation of a Torenia petal cDNA library

A λZAPII (*Eco*RI/*Xho*I directional) kit (Stratagene) was used to prepare a cDNA library from RNA isolated from petals of opening buds of *Torenia hybrida.* cv. Summerwave (Suntory Ltd.) according to the conditions recommended by the manufacturer.

About 200,000 pfus were screened with DIG-labelled *Petunia FMT* (*E20*) cDNA clone from pCGP1907 (Figure 3) using low stringency conditions as described by Tanaka *et al.*, (*Plant Cell Physiol 37*: 711-716, 1996). Twenty hybridizing plaques were picked into PSB. They were rescreened to isolate purified plaques, using the hybridization conditions as described for the initial screening of the cDNA library. The plasmids contained in the λZAPII bacteriophage vector were rescued and sequence data was generated from the 3' and 5' ends of the cDNA inserts. Of these *TFMT* represented the longest cDNA clone (~1 kb) and the plasmid was designated as pTMT5 (Figure 8).

The complete sequence of the *Torenia FMT* cDNA clone (*TFMT*) [SEQ ID NO:11] was determined by compilation of sequence from different pUC18 subclones obtained using standard procedures for the generation of randomly-overlapping clones (Sambrook *et al.*, 1989, *supra*). The sequence was determined to be 1012 bases in length and contains an open reading frame that encodes a putative polypeptide of 240 amino acids [SEQ ID NO:12]. The *TFMT* clone shared 50% identity with the *Petunia E20* sequence [SEQ ID NO:4] at the nucleotide level and 51% identity with the *Petunia E33* sequence [SEQ ID NO:6 and SEQ ID NO:26]. The deduced amino acid sequence of the *Torenia FMT* clone (*TFMT*) shared 56% identity and 70% similarity at the amino acid level with that of the *Petunia* FMT (E20) clone [SEQ ID NO: 5]. The deduced amino acid sequence of the *Torenia FMT* clone (*TFMT*) shared 69% identity and 82% similarity at the amino acid level with that of the *Petunia* FMT (E33-corrected) clone [SEQ ID NO: 7].

Methyltransferase activity of the Torenia FMT cDNA clone expressed in E. coli

The *Torenia FMT* cDNA clone (*TFMT*) was also expressed in an *E. coli* expression system (analogous to that used in Example 7) and assayed for FMT activity.

Cloning of Torenia FMT into pQE30 E. coli expression vector (Construction of pCGP3090)

In order to clone the *Torenia FMT* cDNA clone into an *E. coli* expression vector, pQE30 (QIAGEN), a *Bam*HI restriction endonuclease site was required at the translation initiating ATG and a *Pst*I restriction endonuclease site was required immediately 3' to the putative stop codon.

The oligonucleotides TMT5.*Bam*HI.F [SEQ ID NO:13] and TMT5.*Pst*I.R [SEQ ID NO:14] (Table 14) were used as primers with pTMT5 as template to amplify the *Torenia FMT* cDNA clone with a *Bam*HI restriction endonuclease recognition site in place of the initiating AUG and a *Pst*I restriction endonuclease recognition site immediately 3' to the putative stop codon.

PCR conditions included 5 µL 10 x PfuTurbo DNA Polymerase buffer (Stratagene), 2 µL 10 mM dNTPs, 2 µL 20 µ/µL TMT5.*Bam*HI.F [SEQ ID NO:13], 2 µL 20 µ/µL TMT5.*Pst*I.R [SEQ ID NO:14], 1 µL 1 µg/µL pTMT5 template, 37 µL pure water and 1 µL PfuTurbo DNA Polymerase (Stratagene). The PCR was incubated at 95°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute and then a final incubation at 72°C for 10 minutes with subsequent storage at 4°C.

TABLE 14

Oligonucleotides used in the cloning of the TFMT cDNA clone into pQE30 bacterial expression vector

SEQ ID NO:	PRIMER	SEQUENCE (5' to 3')
13	TMT5.*Bam*HI.F	GCA TGG ATC CAA AGA TAA GTT CTA TGG CAC CAT TTT G
14	TMT5.PstI.R	GCA TCT GCA GTT ATT TGA GAC GTT TGC ACA AGG TG

The resulting PCR products were electrophoresed through a 1% w/v agarose gel and a 0.72 kb band was isolated and purified using a QIAEX II Gel Extraction kit (QIAGEN) according to manufacturer's recommendation. The isolated products were then digested with the restriction endonuclease *Pst*I. The digestion product was purified using a QIAquick PCR purification kit (QIAGEN) and then digested with the restriction endonuclease *Bam*HI. The *Bam*HI/*Pst*I digested products were finally purified using a QIAquick PCR purification kit (QIAGEN) and subsequently ligated with the *Bam*HI/*Pst*I ends of the pQE30 vector (QIAGEN) using DNA Ligation Kit (Amersham) according to the manufacturer's recommendations. Transformants were analyzed for the presence of the specific 0.72 kb insert using *BamHI*/*Pst*I restriction endonuclease digests. The sequence of the insert was confirmed by sequence analysis using pQE Sequencing-Primer Set (QIAGEN). The resulting plasmid was designated pCGP3090 (mut-*TFMT* in pQE30) (Figure 9).

As a consequence of using the TMT5.*Bam*HI.F [SEQ ID NO:13] and TMT5.*Pst*I.R [SEQ ID NO:14] oligonucleotides as primers in the PCR and of the subsequent cloning of the product into pQE30, the sequence of the *Torenia FMT* clone was altered around the putative initiating methionine of the encoded polypeptide. As a consequence the expected amino acids around the putative initiating methionine were changed from "M K D K F Y G T" to "M R G S H H H H H H G S K D K F Y G T".

For analysis of methyltransferase activity of the *Torenia* FMT, the plasmid pCGP3090 was subsequently introduced into *E. coli* M15 ($pREP_4$) (QIAGEN) cells according to the method of Inoue *et al.*, 1990, *supra*.

The confirmation of recombinant protein expression and preparation of crude protein extracts and subsequent determination of methyltransferase activity were as described for the analysis of the *Petunia E20* cDNA clone (*PFMT*) (described above in Example 7).

The enzyme activities of the protein encoded by the *Torenia FMT* cDNA clone in pCGP3090 along with that of the *Petunia FMT* (*E20*) clone in pCGP3086 were assessed using the substrates delphinidin 3-glucoside and delphinidin 3-rutinoside under assay conditions as described in Jonsson *et al.* (1983), *supra* and in Example 7 of this specification.

Methyltransferase assays were set up according to Table 15 in a total reaction volume of 50 µL.

TABLE 15

Composition of methyltransferase assays using crude homogenates from bacterial cultures containing the plasmids pCGP3086 (PFMT) or pCGP3090 (TFMT) or pQE30 (control)

#	Plasmid	Crude homogenate (µL)	3 mg/mL D3R (µL)	3 mg/mL D3G (µL)	SAM (µL)	Buffer (µL)
1	pQE30	20	5	0	5	20
2	pQE30	20	0	5	5	20
3	pQE30	20	5	0	0	25
4	pQE30	20	0	5	-	25
5	pQE30	20	0	-	5	25
6	none	0	5	0	5	40
7	none	0	0	5	5	40

#	Plasmid	Crude homogenate (µL)	3 mg/mL D3R (µL)	3 mg/mL D3G (µL)	SAM (µL)	Buffer (µL)
8	pCGP3086	20	5	0	5	20
9	pCGP3086	20	0	5	5	20
10	pCGP3086	20	5	0	0	25
11	pCGP3086	20	0	5	0	25
12	pCGP3090	20	0	5	5	20
13	pCGP3090	20	0	5	0	25

= tube number,

D3G = delphinidin 3-glucoside,

D3R = delphinidin 3-rutinoside,

^{14}C-SAM = 0.6mM ^{14}C-SAM (13 µCi/µmol) (Amersham Pharmacia),

Buffer = 0.1 M NaPi, pH7.5, 4 mM $MgCl_2$

Reaction conditions were as described previously (Example 7).

TABLE 16

Results of methyltransferase assays of extracts of E. coli containing pCGP3086, pCGP3090 or pQE30 control vector using delphinidin 3-glucoside or delphinidin 3-rutinoside as substrate and ^{14}C-SAM as methyl donor

#	Plasmid	Crude homogenate	D3R	D3G	SAM	Petunidin	Malvidin
1	pQE30	+	+	-	+	no	no
2	pQE30	+	-	+	+	no	no
3	pQE30	+	+	-	-	no	no
4	pQE30	+	-	+	-	no	no
5	pQE30	+	-	-	+	no	no
6	none	-	+	-	+	no	no
7	none	-	-	+	+	no	no

#	Plasmid	Crude homogenate	D3R	D3G	SAM	Petunidin	Malvidin
8	pCGP3086	+	+	-	+	yes	yes
9	pCGP3086	+	-	+	+	yes	yes
10	pCGP3086	+	+	-	-	no	no
11	pCGP3086	+	-	+	-	no	no
12	pCGP3090	+	-	+	+	yes	yes
13	pCGP3090	+	-	+	-	no	no

# = Tube number,
D3G = delphinidin 3-glucoside,
D3R = delphinidin 3-rutinoside,
^{14}C-SAM = ^{14}C-labelled *S*-adenosyl-L-methionine (Amersham Biosciences),
\+ = present in reaction mix
\- = absent from reaction mix
yes = detection of product on TLC,
no = no reaction observed as determined by absence of product.

Petunidin and malvidin, the methylated derivatives of delphinidin, were detected in assay reactions using crude homogenates from pCGP3090 (containing *TFMT*) and D3G (Tube 12, Table 15). There was no detectable production of petunidin and malvidin in assay reactions using crude homogenates from pQE30 cells (Tubes 1 to 5, Table 15) or having no crude homogenates added (Tubes 6 and 7, Table 15) or in assay reactions without the addition of ^{14}C-SAM (Tubes 10, 11 and 13, Table 15). The crude homogenates from pCGP3086 (containing PFMT) were used as positive controls (Tubes 8 and 9, Table 16).

The results obtained with expression of the *Torenia FMT* cDNA clone (*TFMT*) in an *E. coli* expression system provide further evidence to suggest that the *TFMT* cDNA clone codes for an FMT that is able to methylate delphinidin 3-glucoside using SAM as a methyl donor to produce the 3'-methylated derivative, petunidin and the 3' 5' methylated derivative, malvidin.

EXAMPLE 10

HPLC assay of methyltransferase activity of Petunia and Torenia FMT clones

The enzyme activities of the peptides encoded by *Petunia* and *Torenia FMT* cDNA clones in pCGP3086 and pCGP3090, respectively were further assessed using the substrates delphinidin 3-glucoside and delphinidin 3-rutinoside and delphinidin 3, 5-diglucoside under assay conditions as previously described (Table 15, Example 9) except that the ^{14}C-labelled SAM was replaced with non radioactive SAM at 2 mg/mL and the substrates (delphinidin 3-glucoside and delphinidin 3-rutinoside and delphinidin 3, 5-diglucoside) at 2 mg/mL.

TABLE 17

Identification of products (in mg/g) by HPLC from methyltransferase assays of extracts of E. coli containing pCGP3086, pCGP3090 or pQE30 control vector using delphinidin 3-glucoside, delphinidin 3-rutinoside and delphinidin 3, 5-diglucoside as substrate and SAM as methyl donor

Tube No.	Substrate	Plasmid	Anthocyanidins (mg/g)					Predominant FMT Activity
			Del	Cya	Pet	Peo	Mal	
1a	D3R	none	17.6	0.6	0	0	0	none
1b			19.9	0.6	0.6	0	0	
2a	D3R	pQE30	16.9	0.6	0.6	0	0	none
2b			21.9	0.6	0.6	0	0	
3a	D3R	pCGP3086	3.7	0.4	10.2	0.2	2.9	3'FMT
3b			4.5	0.5	11.9	0.2	3.6	
4a	D3R	pCGP3090	2.5	0	0.8	0.4	15.6	3'5'FMT
4b			2.8	0	0.8	0.4	15.1	
5a	D3G	none	7.8	1.7	0.9	0	0	none
5b			9.8	1.9	0.9	0	0	
6a	D3G	pQE30	17.1	2.5	1.2	0	0	none
6b			22.1	2.7	1.3	0	0	

Tube No.	Substrate	Plasmid	Anthocyanidins (mg/g)					Predominant FMT Activity
			Del	Cya	Pet	Peo	Mal	
7a	D3G	pCGP3086	7.1	2.4	8.8	0.5	1.2	3'FMT
7b			6.4	2.0	10.0	0.6	1.3	
8a	D3G	pCGP3090	1.8	1.0	1.2	2.0	17.1	3'5'FMT
8b			1.9	0.9	1.3	1.9	18.1	
9a	D3,5G	none	4.2	0	0	0	0	none
9b			17.1	0	0	0	0	
10a	D3,5G	pQE30	5.3	0	0	0	0	none
10b			16.0	0	0	0	0	
11a	D3,5G	pCGP3086	2.9	0	2.7	0	0.7	3'FMT
11b			10.5	0.4	6.7	0	1.1	
12a	D3,5G	pCGP3090	2.4	0	0.7	0	7.3	3'5'FMT
12b			5.4	0	0.8	0	12.4	

Tube No. = Tube numbers ("a" and "b" refer to duplicate product measurements)

3'FMT = flavonoid 3' methyltransferase,

3'5'FMT = flavonoid 3' 5' methyltransferase,

Del = delphinidin,

Cya = cyanidin,

Pet = petunidin,

Peo = peonidin,

Mal = malvidin.

TABLE 18

Products (expressed as percentage of total anthocyanidin) of the methyltransferase assays of extracts of E. coli containing pCGP3086, pCGP3090 or pQE30 control vector using delphinidin 3-glucoside, delphinidin 3-rutinoside and delphinidin 3,5-diglucoside as substrate and SAM as methyl donor

Tube No.	Substrate	Plasmid	% of anthocyanidin detected				
			del	cya	pet	peo	mal
1a	D3R	none	97.0	3.0	0.0	0.0	0.0
1b			94.3	2.7	3.0	0.0	0.0
2a	D3R	pQE30	93.2	3.4	3.4	0.0	0.0
2b			94.7	2.5	2.8	0.0	0.0
3a	**D3R**	**pCGP3086**	**21.2**	**2.5**	**58.5**	**1.2**	**16.6**
3b		***(PFMT)***	**21.7**	**2.5**	**57.4**	**1.1**	**17.4**
4a	**D3R**	**pCGP3090**	**13.2**	**0.0**	**4.0**	**1.9**	**80.9**
4b		***(TFMT)***	**14.6**	**0.0**	**4.3**	**1.9**	**79.1**
5a	D3G	none	75.3	16.5	8.2	0.0	0.0
5b			77.7	15.2	7.1	0.0	0.0
6a	D3G	pQE30	82.5	12.0	5.6	0.0	0.0
6b			85.0	10.2	4.8	0.0	0.0
7a	**D3G**	**pCGP3086**	**35.5**	**11.8**	**44.0**	**2.7**	**6.0**
7b		***(PFMT)***	**31.4**	**9.7**	**49.4**	**3.0**	**6.5**
8a	**D3G**	**pCGP3090**	**7.8**	**4.5**	**5.1**	**8.5**	**74.0**
8b		***(TFMT)***	**7.8**	**3.6**	**5.4**	**8.0**	**75.2**
9a	D3,5G	non	100.0	0.0	0.0	0.0	0.0
9b			100.0	0.0	0.0	0.0	0.0
10a	D3,5G	pQE30	100.0	0.0	0.0	0.0	0.0
10b			100.0	0.0	0.0	0.0	0.0
11a	**D3,5G**	**pCGP3086**	**45.6**	**0.0**	**43.4**	**0.0**	**11.0**
11b		***(PFMT)***	**55.9**	**2.2**	**35.9**	**0.0**	**6.0**
12a	**D3,5G**	**pCGP3090**	**22.9**	**0.0**	**7.1**	**0.0**	**70.0**

Tube No.	Substrate	Plasmid	% of anthocyanidin detected				
			del	cya	pet	peo	mal
12b		(*TFMT*)	28.9	0.0	4.3	0.0	66.9

Tube No. = Tube numbers ("a" and "b" refer to duplicate product measurements)
del = delphinidin, expressed as a percentage of total anthocyanidins detected,
cya = cyanidin, expressed as a percentage of total anthocyanidins detected,
pet = petunidin, expressed as a percentage of total anthocyanidins detected,
peo = peonidin, expressed as a percentage of total anthocyanidins detected,
mal = malvidin, expressed as a percentage of total anthocyanidins detected.

Under the conditions of the assay, the *Petunia FMT* (*E20*) cDNA clone contained in pCGP3086 led to a flavonoid methyltransferase activity that utilized delphinidin 3-glucoside, delphinidin 3-rutinoside or delphinidin 3, 5-diglucoside as substrate to produce predominantly petunidin and to a lesser degree, malvidin.

Previously published data on methyltransferase activities in crude protein extracts of petunia flowers suggest that the *Petunia* methyltransferases cannot utilize anthocyanidin 3-glucoside or anthocyanidin 3-rutinosides as substrates (Jonsson *et al.*, 1982, *supra*). Under our assay conditions, however, the *Petunia* methyltransferase activity produced by the *Petunia E20* clone in pCGP3086 was able to methylate each of delphinidin 3-glucoside, delphinidin 3-rutinoside and delphinidin 3,5-diglucoside.

The *Torenia FMT* cDNA clone contained in pCGP3090 also resulted in a flavonoid methyltransferase activity that utilized delphinidin 3-glucoside, delphinidin 3-rutinoside and delphinidin 3, 5-diglucoside as substrate to produce predominantly malvidin and to a lesser degree, petunidin.

EXAMPLE 11

Transformation of rose to produce malvidin-based pigments.

The predominant anthocyanins in commercially grown roses tend to be 3-glucosides or 3, 5-diglucosides of cyanidin or pelargonidin (Mikanagi *et al., Biochem. System and Ecol. 23:* 183-200, 1995, Mikanagi *et al., Biochem. System and Ecol. 28*: 887-902, 2000). In order to produce malvidin-based pigments in these roses, a *F3'5'H* gene would need to be introduced to initially produce the precursors of malvidin pigments, delphinidin 3-glucosides or delphinidin 3, 5 diglucosides. To then allow for conversion to malvidin pigments, a flavonoid methyltransferase with 3' and 5' activity and the ability to utilize 3-glucosides or 3, 5-diglucosides of delphinidin would be required.

The binary vector plasmids pCGP3254 (Figure 13), pSPB1534 (Figure 15) and pSPB1532 (Figure 18) containing a *F3'5'H* chimaeric gene along with a *Petunia* or *Torenia FMT* genes were, therefore, constructed to be introduced into rose to allow for the production of petunidin and/or malvidin-based pigments and thereby modify flower color. These binary plasmids are also introduced into a species that does not normally produce delphinidin-based pigments and does not contain a flavonoid methyltransferase capable of methylating anthocyanidins, specifically delphinidin. Such plants may include but are not limited to carnation, chrysanthemum, gerbera, orchids, *Euphorbia, Begonia.*

Construction of the binary vector pCGP3254

(35S 5': TFMT: 35S 3'; 35S 5': Viola F3'5'H: 35S 3'; 35S 5': SuRB)

The plasmid pCGP3254 contains a *35S 5': Viola F3'5'H: 35S 3'* expression cassette (from pCGP2092) (Figure 14) and a *35S 5': Torenia FMT: 35S 3'* expression cassette (from pCGP3099) (Figure 11) in tandem orientation with the selectable marker gene of the Ti binary vector pCGP1988 (Figure 12).

(1) Construction of intermediate plasmids to pCGP3254

(i) Construction of pCGP3097 (35S 5': TFMT: 35S 3' expression cassette)

Plasmid pCGP3097 (Figure 10) was constructed by cloning the *Torenia FMT* cDNA clone from pTMT5 into a *CaMV 35S* expression cassette.

The plasmid pRTppoptcAFP was used as a source of a *CaMV 35S* promoter and terminator fragment. It was initially digested with *Xba*I, the overhanging 5' ends were repaired and then the plasmid was restricted with *Eco*RI to release the 3.3kb vector containing the *CaMV 35S* expression cassette. The 3.3 kb vector was isolated and purified.

pTMT5 was digested initially with the restriction endonuclease *Asp*718 and the resulting 5' overhang ends were repaired. The linearized plasmid was then restricted with the restriction endonuclease *Eco*RI to release the 1.0kb *Torenia FMT* cDNA fragment which was isolated, purified and then ligated with the *Xba*I (blunt)/ *Eco*RI ends of the pRTppoptc vector (described above). Correct ligation of the fragments was established by restriction endonuclease analysis (*Hin*DIII, *Cla*I, *Xho*I, *Pst*I, *and SphI*) of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated pCGP3097 (Figure 10).

(ii) Construction of pCGP3099 (35S 5': TFMT: 35S 3'; 35S 5': SuRB expression binary)

Plasmid pCGP3099 (Figure 11) was constructed by cloning the chimaeric *Torenia FMT* gene from pCGP3097 (Figure 10) into the Ti binary vector pCGP1988. The binary vector pCGP1988 (Figure 12) is based on the binary vector pWTT2132 (DNAP) but contains the multi-cloning site from pNEB193 (New England Biolabs).

The *35S 5': Torenia FMT: 35S 3'* expression cassette from pCGP3097 (Figure 10) was released by digestion with the restriction endonuclease *Pst*I. A 1.66 kb fragment containing the chimaeric *Torenia FMT* gene was subsequently isolated and ligated with *Pst*I ends of

pCGP1988. Correct ligation of the chimaeric gene in tandem with the *35S 5': SuRB* gene of pCGP3099 was established by restriction endonuclease analysis (*Hin*DIII, *Xho*I, *Pst*I, *Asp*718, *Eco*RI, *and EcoRV*) of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP3099 (Figure 11).

(iii) Construction of pCGP2092 (35S 5': Viola F3'5'H (BP#40): 35S 3' expression cassette)

The plasmid pCGP2092 (Figure 14) was constructed by cloning the *F3'5'H* cDNA clone isolated from *Viola sp.* as a *Xba*I/*Eco*RI 1.6 kb fragment from pCGP1961 behind the *CaMV 35S* promoter contained in pRTppoptc.

The plasmid pCGP1961 (Australian Provisional Patent Applications No. 2002951088 and 2002952835, 2002, *supra*) was initially digested with the restriction endonuclease *Asp*718 and after repair of the overhanging 5' ends was digested with the restriction endonuclease *Eco*RI to release a 1.6 kb fragment containing the *F3'5'H* chimaeric gene. The fragment was isolated and ligated with *Xba*I (blunt)/*Eco*RI ends of the 3.3kb pRTppoptc vector (described above). Correct ligation of the *Viola F3'5'H* cDNA clone (*BP#40*) into the *CaMV 35S* expression cassette was established by restriction endonuclease analysis (*Hin*DIII, *Xho*I, *Pst*I) of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated as pCGP2092 (Figure 14).

Construction of pCGP3254

The chimaeric *F3'5'H* gene was subsequently released from pCGP2092 by restriction with the restriction endonuclease *Pst*I followed by treatment with T4 DNA polymerase to repair the overhanging 3' ends. The fragment was isolated and ligated with the *Sma*I ends of pCGP3099 (described above). Correct insertion of the *F3'5'H* chimaeric gene in tandem with the *35S 5': SuRB* gene and *35S 5': Torenia FMT: 35S 3'* expression cassette gene was established by restriction endonuclease analysis (*Hin*DIII, *Xho*I, *Nco*I, *Sal*I, *Eco*RI, *Eco*RV)

of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP3254 (Figure 13).

Plant transformation with pCGP3254

The binary vector pCGP3254 (Figure 13) was introduced into *A. tumefaciens* strain AGL0 and the T-DNA contained in pCGP3254 was subsequently introduced into rose cultivars Medeo and Sonia via *Agrobacterium*-mediated transformation.

(2) Construction of the binary vectors

(a) pSPB1534 (e35S 5': BP#40: pet D8 3'; e35S 5': PFMT: nos 3') and

(b) pSPB1532 (e35S 5': BP#40: pet D8 3'; e35S 5': TFMT: nos 3')

(a) The binary vector plasmid pSPB1534 (Figure 15) contains an *e35S 5': Viola F3'5'H (BP#40): pet D8 3'* expression cassette (from pSPB580 (Figure 16)) in tandem orientation with an *e35S: PFMT: nos 3'* expression cassette (from pSPB1531 (Figure 17)). Both chimaeric genes are in a tandem orientation with the *nos 5': nptII: nos 3'* selectable marker gene cassette of the Ti binary vector pBINPlus (van Engelen *et al.*, *Transgenic Research*, 4: 288-290, 1995).

(b) The binary vector plasmid pSPB1532 (Figure 18) contains an *e35S 5': Viola F3'5'H (BP#40): pet D8 3'* expression cassette (from pSPB580 (Figure 16)) in tandem orientation with an *e35S 5': TFMT: nos 3'* expression cassette (from pSPB1530 (Figure 19)). Both chimaeric genes are in a tandem orientation with the *nos 5': nptII: nos 3'* selectable marker gene cassette of the Ti binary vector pBINPlus (van Engelen *et al.*, 1995, *supra*).

TABLE 19

Oligonucleotides used as primers in the construction of the binary vectors pSPB1534 and pSPB1532

SEQ ID NO:	NAME	SEQUENCE (5' TO 3')
28	petD8 #1	CCC TCG AGT TTC TAT TTT GTG TGT GTT G
29	petD8 #2	GGG AAT TCT AGA GCT CGA GGA TCA CG
30	PMT-F	ACT ACC AAG GAT CCT ACT GAA GCA
31	PMT-R	CTC GAA TGA AGC TTT TGT TA
32	TMT-F	CAT AAA TAG GAT CCG CAG CAG CAA
33	TMT-R	AGT CTC ATA AGC TTC TCT AT

Construction of intermediate plasmids to pSPB1534 and pSPB1532

(i) Construction of pSPB580 (e35S 5': BP#40: pet D8 3')

The plasmid pSPB580 (Figure 16) contains the *Viola F3'5'H* cDNA clone in between an enhanced *CaMV 35S* promoter fragment (*e35S 5'*) and a *Petunia PLTP* terminator (*petD8 3'*) fragment.

(1) Isolation of the F3'5'H clone from Viola sp.

The isolation of a *F3'5'H* cDNA clone from *Viola sp.* cultivar black pansy has been described in Australian Provisional Patent Application Nos. 2002951088 and 2002952835, *supra*). The plasmid pCGP1961 (Australian Provisional Patent Application Nos. 2002951088 and 2002952835, *supra*) was linearized upon digestion with the restriction endonuclease *Bam*HI. An ~1.7 kb DNA fragment containing a *F3'5'H* cDNA clone (*BP#40*) from *Viola sp.* cv. black pansy was recovered upon partial digestion with the restriction endonuclease, *Xho*I.

(2) Isolation of an enhanced CaMV 35S promoter fragment

The binary vector, pBE2113-GUS contains a *GUS* gene under the control of an enhanced *CaMV 35S* promoter (*e35S 5'*) with a terminator region from the nopaline synthase gene of *Agrobacterium* (*nos 3'*) (Mitsuhashi *et al., Plant Cell Physiol. 37*: 49-59, 1996). The plasmid pBE2113-GUS was digested with the restriction endonuclease *Sna*BI and a *Bam*HI linker (5'-GGGATCCC-3') [SEQ ID NO:45] was then ligated with the overhanging ends to yield pBE2113-ΔGUS. A ~0.7 kb fragment containing the enhanced *CaMV 35S* promoter (*e35S 5'*) was then released upon digestion of pBE2113-ΔGUS with the restriction endonucleases *Hin*dIII and *Bam*HI.

(3) Isolation of a terminator fragment from the Petunia PLTP (D8) gene (petD8 3')

A terminator fragment from the *Petunia* phospholipid transfer protein (*PLTP*) gene (*petD8 3'*) (Holton, 1992, *supra*) was amplified by PCR. The primers pet D8 #1 [SEQ ID NO: 28] (Table 19) and pet D8 #2 [SEQ ID NO: 29] (Table 19) along with the plasmid template pCGP13ΔBam (Holton, 1992, *supra*) were used to amplify the ***Petunia*** *PLTP* terminator fragment (*petD8 3'*). The amplified fragment of about 0.8 kb was then digested with the restriction endonucleases *Eco*RI and *Xho*I.

(4) Construction of pUCAPAsc- (a shuttle cloning vector)

The plasmid pUCAP is based on the cloning vector pUC19 (NEB) but contains an extended multiple cloning site (VanEngelen *et al., Transgenic Res.4*: 288-290, 1995). pUCAP was digested with the restriction endonuclease *Pac*I. The overhanging ends were repaired and then ligated with *Asc*I linker (5'-GGCGCGCC-3') [SEQ ID NO:46] to yield pUCAPAsc (similar to pUCAP without a *Pac*I recognition site and with 2 *Asc*I recognition sequences at either ends of the multiple cloning site).

(5) Construction of pSPB580 (e35S: BP#40: pet D8 3')

The 1.7 kb *Bam*HI/*Xho*I fragment containing the *Viola F3'5'H* (*BP#40*) cDNA clone (isolation described above) was ligated with the *Bam*HI/*Eco*RI 2.7 kb vector fragment obtained from pUCAPAsc (described above) and the *Eco*RI/*Xho*I fragment containing the a *Petunia PLTP* terminator (*petD8 3'*) (described above). Correct insertion of the fragments was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated pSPB51

The 0.7 kb *Hin*dIII/*Bam*HI fragment containing the enhanced *CaMV 35S* promoter region (described above) was ligated with the *Hin*dIII/*Bam*HI ends of the plasmid pSPB51. Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated pSPB580 (Figure 16).

(ii) *Construction of the binary vector pSPB176 (e35S 5': GUS: nos 3'; nos 5': nptII: nos 3')*

The binary vector pSPB176 (Figure 20) contains an *e35S 5': GUS: nos 3'* expression cassette in a tandem orientation to the selectable marker gene cassette of the Ti binary vector pBINPlus (van Engelen *et al.*, 1995, *supra*).

The plasmid pBE2113-ΔGUS (described above) was digested with *Sac*I. The overhanging 3' ends were repaired and then ligated with a *Sal*I linker (5'-GGTCGACC-3') [SEQ ID NO:47]to yield pBE2113-ΔGUSs. A fragment containing the *e35S 5': GUS: nos 3'* expression cassette was released from pBE2113-ΔGUSs upon digestion with the restriction endonucleases *Hin*dIII and *Eco*RI. The *Hin*dIII/*Eco*RI fragment was then ligated with *Hin*dIII/*Eco*RI ends of the Ti binary vector pBinPLUS (VanEngelen *et al.*, 1995, *supra*). Correct insertion of the fragment was established by restriction endonuclease analysis of plasmid DNA isolated from kanamycin-resistant transformants. The resulting plasmid was designated pSPB176 (Figure 20).

(iii) ***Construction of the intermediate binary vector pSPB1531 (e35S 5': PFMT: nos 3'; nos 5': nptII: nos 3')***

The binary vector plasmid pSPB1531 (Figure 17) contains the *Petunia FMT* cDNA clone (with a shortened 5' non-coding region as compared to the *E20* clone) between an enhanced *CaMV 35S* promoter fragment (*e35S 5'*) and a *nos* terminator fragment (*nos 3'*) in tandem with the *nos 5': nptII: nos 3'* selectable marker gene cassette of the Ti binary vector pBINPlus (van Engelen *et al.*, 1995, *supra*).

The 5' region of the *Petunia FMT* cDNA clone contained in pCGP1907 (Figure 3) was amplified by PCR using the primers PMT-F [SEQ ID NO: 30] and PMT-R [SEQ ID NO: 31] and 10 ng of the plasmid pCGP1907 as template. The oligonucleotide PMT-F [SEQ ID NO:30] was designed to amplify from position 43-66) of SEQ ID NO:4 and incorporated a *Bam*HI recognition sequence for ease of cloning. The PMT-R [SEQ ID NO: 31] primer was designed to amplify from position 192-173 of SEQ ID NO:4 and incorporated a *Hin*dIII recognition sequence for ease of cloning. The amplified *Petunia FMT* 5' partial fragment was then digested with the restriction endonucleases *Bam*HI and *Hin*dIII and ligated with the 0.7 kb *Hin*dIII/*Xho*I *Petunia FMT* 3' partial fragment isolated from the plasmid pCGP1907 (Figure 3) and *Bam*HI/*Sal*I ends of the Ti binary vector pSPB176 (Figure 20). Correct insertion of the fragments was established by restriction endonuclease analysis of plasmid DNA isolated from kanamycin-resistant transformants. The resulting plasmid was designated pSPB1531 (Figure 17).

(iv) ***Construction of the intermediate binary vector pSPB1530 (e35S 5': TFMT: nos 3'; nos 5': nptII: nos 3')***

The binary vector plasmid pSPB1530 (Figure 19) contains the *Torenia FMT* cDNA clone (with a shortened 5' non-coding region as compared to the *TFMT* clone) between an enhanced *CaMV 35S* promoter fragment (*e35S 5'*) and a *nos* terminator fragment (*nos 3'*)

in tandem with the *nos 5': nptII: nos 3'* selectable marker gene cassette of the Ti binary vector pBINPlus.

The 5' region of the *Torenia FMT* cDNA clone contained in pTMT5 was amplified by PCR using the primers TMT-F [SEQ ID NO:32] and TMT-R [SEQ ID NO:33] (Table 19) and 10 ng of pTMT5 as the template. The oligonucleotide TMT-F [SEQ ID NO:32] (Table 19) was designed to amplify from position 34-53 of SEQ ID NO:11 and incorporated a *Bam*HI recognition sequence for ease of cloning. The TMT-R [SEQ ID NO: 33] (Table 19) primer was designed to amplify from position 214-190 of SEQ ID NO:11 and incorporated a *Hin*dIII recognition sequence for ease of cloning. The amplified *Torenia FMT* 5' partial fragment was then digested with the restriction endonucleases *Bam*HI and *Hin*dIII and ligated with a ~0.6 kb *Hin*dIII/*Xho*I *Torenia FMT* 3' partial fragment isolated from pTMT5 and *Bam*HI/*Sal*I ends of the Ti binary vector pSPB176 (Figure 20). Correct insertion of the fragments was established by restriction endonuclease analysis of plasmid DNA isolated from kanamycin-resistant transformants. The resulting plasmid was designated pSPB1530 (Figure 19).

(v) Construction of the binary vector pSPB1534 (e35S 5': BP#40: pet D8 3'; e35S 5': PFMT: nos 3'; nos 5': nptII: nos3')

An ~3.1kb DNA fragment containing the *e35S 5': Viola F3'5'H (BP40): pet D8 3'* expression cassette was isolated from the plasmid pSPB580 (Figure 16) upon digestion with the restriction endonuclease *Asc*I. The purified fragment was ligated with the *Asc*I ends of the Ti binary plasmid pSPB1531 (Figure 17). Correct insertion of the fragment in a tandem orientation with the *Petunia FMT* cassette and the selectable marker cassette was established by restriction endonuclease analysis of plasmid DNA isolated from kanamycin-resistant transformants. The resulting plasmid was designated pSPB1534 (Figure 15).

Plant transformation with pSPB1534

The binary vector plasmid pSPB1534 (Figure 15) was introduced into *A. tumefaciens* strain AGL0 and the T-DNA contained in pSPB1534 was introduced into the *Rosa hybrida* cultivar WKS124 *via Agrobacterium*-mediated transformation.

Construction of the binary vector pSPB1532 (e35S 5': BP#40: pet D8 3'; e35S 5': TFMT: nos 3'; nos 5': nptII: nos 3')

An ~3.1kb DNA fragment containing *e35S: Viola F3'5'H (BP#40): pet D8 3'* cassette was isolated from the plasmid pSPB580 (Figure 16) upon digestion with the restriction endonuclease *Asc*I. The purified fragment was ligated with the *Asc*I ends of the Ti binary plasmid pSPB1530 (Figure 19). Correct insertion of the fragment in a tandem orientation with the *Torenia FMT* cassette and the selectable marker cassette was established by restriction endonuclease analysis of plasmid DNA isolated from kanamycin-resistant transformants. The resulting plasmid was designated pSPB1532 (Figure 18).

Plant transformation with pSPB1532

The binary vector plasmid pSPB1532 (Figure 18) was introduced into *A. tumefaciens* strain AGL0 and the T-DNA contained in pSPB1532 was introduced into *Rosa hybrida* cultivars Lavande and WKS124 *via Agrobacterium*-mediated transformation.

Transgenic Analysis of rose petals

Independent transgenic plants were produced and grown to flowering (Table 20). Petal flower color was measured with the spectrophotometer CM-2002 (Minolta, Japan) installed with the software SpectraMagic (Minolta, Japan) in order to obtain its hue and reflectance (Tables 21, 22 and 23). Hue (0-360°) is the basic color of an object such as red, green, purple, etc., and is defined by its angular position in a cylindrical color space, or on a Color Wheel. Pure red and blue are 0 and 270 degrees, respectively. The closer the hue is

to 270°, the bluer the color. Reflectance (%) is the percentage of light that is reflected from an object. Spectrophotometers measure an object's reflectance at various intervals along the visible spectrum to determine the object color's spectral curve. A lower reflectance value suggests a darker color. Royal Horticultural Society Colour Charts (RHSCC) were also used to define the color of the petals (Tables 21, 22 and 23). RNA blot analysis was performed on a selection of flowers to confirm the presence of the transgenic transcripts. HPLC analysis of the anthocyanidins accumulating in the petals of the transgenic roses was used to detect the production of the novel anthocyanins, petunidin and malvidin in rose flowers (Tables 21, 22 and 23).

TABLE 20

Number of independent transgenic rose events produced from transformation with T-DNAs contained in the plasmids pCGP3254, pSPB1532 and pSPB1534'

Cultivar	Color	Plasmid	Genes	#	# flowered	# mod col
Sonia	Apricot	pCGP3254	*F3'5'H* and *TFMT*	36	8	5
Medeo	Pale apricot	pCGP3254	*F3'5'H* and *TFMT*	2	0	na
Lavande	Pink	pSPB1532	*F3'5'H* and *TFMT*	140	126	30
WKS124	Apricot	pSPB1532	*F3'5'H* and *TFMT*	90	75	75
WKS124	Apricot	pSPB1534	*F3'5'H* and *PFMT*	60	48	46

refers to the number of independent transgenic events produced

#flowered refers to the number of independent events that have flowered to date

#mod col refers to the number of independent transgenic events producing flowers with a modified petal color compared to the control

Anthocyanins of the flowers of the transgenic roses were extracted and the anthocyanidins derived from the anthocyanins were analyzed by HPLC system as described in Fukui *et al.*, (*Phytochemistry, 47:* 1409-1416, 1998). The methylated derivatives of delphinidin, malvidin and petunidin were detected in a number of flowers of transgenic roses with

modified flower color (Tables 21, 22 and 23). Peonidin, the methylated derivative of cyanidin was also detected in the flowers of transgenic roses (Tables 21, 22 and 23).

TABLE 21

Levels of the anthocyanidins detected in a selection of flowers from independent transgenic events of R. hybrida cv. WKS124 transformed with the T-DNA in pSPB1532 containing Viola F3'5'H and Torenia FMT chimaeric genes

code	Del (mg/g)	Cya (mg/g)	Pet (mg/g)	Pel (mg/g)	Peo (mg/g)	Mal (mg/g)	Total (mg/g)	hue	ref (%)	DPM (%)	Mal (%)	Methyl (%)	RHSCC	Petal Color
5-1	0.22	0.02	0.18	0.07	0.06	0.64	1.19	341.86	3.51	92	57	78	72a	purple
5-2	0.20	0.03	0.18	0.01	0.10	0.80	1.32	341.31	2.32	89	60	82	72a	red-purple
7-1	0.45	0.07	0.32	0.01	0.14	0.82	1.81	352.50	0.89	88	45	71	61a	red-purple
7-4	0.22	0.02	0.19	0.00	0.11	1.02	1.56	345.43	1.45	91	65	84	72a	purple
12-1	0.13	0.01	0.11	0.00	0.07	0.86	1.18	343.56	1.49	93	73	88	78a	purple
12-2	0.14	0.01	0.14	0.00	0.06	1.12	1.47	347.08	0.84	95	76	90	78a	purple
12-3	0.22	0.03	0.16	0.00	0.04	0.27	0.72	346.05	2.96	90	37	64	64b	red-purple
25-1	0.22	0.01	0.19	0.00	0.09	0.83	1.34	345.03	0.79	92	62	83	78a	purple
25-2	0.25	0.01	0.19	0.00	0.06	1.24	1.3	342.77	0.87	96	71	85	78a	purple
control	0	0.01	0	0.07	0	0	0.08	31.14	30.81	0	0	0	38b	apricot

Code = the accession number of the transgenic plant,
Del, Cya, Pet, Pel, Peo, Mal (mg/g) refer to the amount of the specific anthocyanidin detected in mg/g where Del = delphinidin, Cya = cyanidin, Pet = petunidin, Pel = pelargonidin, Peo = peonidin, Mal = malvidin
DPM (%) = delphinidin or its methylated derivatives, petunidin and malvidin expressed as a percentage of total anthocyanidins detected
Mal (%) = malvidin expressed as a percentage of total anthocyanidins detected
Methyl (%) = methylated anthocyanidins (petunidin, peonidin, malvidin) expressed as a percentage of total anthocyanidins detected
Total = the total amounts of anthocyanidins detected (delphinidin, petunidin, malvidin, cyanidin, peonidin, pelargonidin) in mg/g
RHSCC = colors observed described according to the Royal Horticultural Society Color Charts
hue = describes the basic color in degrees as measured by a spectrophotometer with SpectraMagic software (Minolta, Japan)
Ref (%) = describes the percentage of light reflected as measured by a spectrophotometer with SpectraMagic software (Minolta, Japan)

TABLE 22

Levels of the anthocyanidins detected in a selection of flowers from independent transgenic events of R. hybrida cv. Lavande transformed with the T-DNA in pSPB1532 containing Viola F3'5'H and Torenia FMT chimaeric genes

code	Del (mg/g)	Cya (mg/g)	Pet (mg/g)	Pel (mg/g)	Peo (mg/g)	Mal (mg/g)	Total (mg/g)	hue (°)	ref (%)	DPM (%)	Mal (%)	Methyl (%)	RHSCC	Petal color
13-3	0.13	0.03	0.04	0	0.01	0.04	0.25	333.81	19.99	82	15	36	75a	pale purple
13-5	0.09	0.01	0.04	0	0.02	0.17	0.33	333.32	10.44	90	52	70	77b	dark purple
17-1	0.11	0.05	0.04	0	0.07	0.09	0.36	344.08	13.07	67	26	57	186b	pink
17-2	0.04	0.02	0.02	0	0.04	0.05	0.17	343.90	17.50	65	30	65	186c	pink
24-2	0.02	0.02	0.01	0	0.03	0.03	0.11	333.23	29.49	63	31	66	77d	pale purple
34-1	0	0.04	0	0	0.08	0	0.12	339.82	17.45	0	0	68	186c	pink
LA control	0	0.08	0	0	0	0	0.08	345.25	16.75	0	0	0	186c	pink

Code = the accession number of the transgenic plant,
Del, Cya, Pet, Pel, Peo, Mal (mg/g) refer to the amount of the specific anthocyanidin detected in mg/g where Del = delphinidin, Cya = cyanidin, Pet = petunidin, Pel = pelargonidin, Peo = peonidin, Mal = malvidin
DPM (%) = delphinidin or its methylated derivatives, petunidin and malvidin expressed as a percentage of total anthocyanidins detected
Mal (%) = malvidin expressed as a percentage of total anthocyanidins detected
Methyl (%) = methylated anthocyanidins (petunidin, peonidin, malvidin) expressed as a percentage of total anthocyanidins detected
Total = the total amounts of anthocyanidins detected (delphinidin, petunidin, malvidin, cyanidin, peonidin, pelargonidin) in mg/g
RHSCC = colors observed described according to the Royal Horticultural Society Color Charts
hue = describes the basic color in degrees as measured by a spectrophotometer with SpectraMagic software (Minolta, Japan)
Ref (%) = describes the percentage of light reflected as measured by a spectrophotometer with SpectraMagic software (Minolta, Japan)

TABLE 23

Levels of the anthocyanidins detected in a selection of flowers from independent transgenic events of R. hybrida cv. WKS124 transformed with the T-DNA in pSPB1534 containing Viola F3'5'H and Petunia FMT chimaeric genes

code	Del (mg/g)	Cya (mg/g)	Pet (mg/g)	Pel (mg/g)	Peo (mg/g)	Mal (mg/g)	Total (mg/g)	hue (°)	ref (%)	DPM (%)	Mal (%)	Methyl (%)	RHSCC	Petal Color
02-2	0.44	0.06	0.01	0.02	0	0	0.53	347.53	10.15	85	0.0	2	64c	red-purple
02-3	0.59	0.09	0.01	0.01	0	0	0.7	346.64	8.51	85	0.0	2	64c	red-purple
07-1	0.91	0.10	0.02	0.03	0	0	1.06	352.44	7.26	87	0.0	2	71b	red-purple
08-3	1.76	0.06	0.03	0.00	0	0	1.85	350.34	1.56	97	0.0	2	61b	red-purple
08-6	1.34	0.06	0.03	0	0	0	1.43	354.15	1.11	96	0.0	2	61a	red-purple
08-7	1.22	0.11	0.02	0.01	0	0	1.36	351.48	1.55	92	0.0	2	64b	red-purple
11-2	1.60	0.17	0.02	0.01	0	0	1.80	357.44	1.14	90	0.0	1	61a	red-purple
14-3	1.35	0.10	0.03	0.00	0	0	1.48	352.54	2.58	93	0.0	2	64b	red-purple
14-5	1.11	0.04	0.03	0	0	0	1.18	352.52	1.78	97	0.0	2	64b	red-purple
15-1	1.04	0.04	0.02	0	0	0	1.10	351.85	1.72	97	0.0	2	64b	red-purple
15-2	1.25	0.06	0.03	0	0	0	1.34	347.89	3.77	96	0	2	64b	red-purple
control	0	0.01	0	0.07	0	0	0.08	31.14	30.81	0	0	0	38b	apricot

Code = the accession number of the transgenic plant,
Del, Cya, Pet, Pel, Peo, Mal (mg/g) refer to the amount of the specific anthocyanidin detected in mg/g where Del = delphinidin, Cya = cyanidin, Pet = petunidin, Pel = pelargonidin, Peo = peonidin, Mal = malvidin
DPM (%) = delphinidin or its methylated derivatives, petunidin and malvidin expressed as a percentage of total anthocyanidins detected
Mal (%) = malvidin expressed as a percentage of total anthocyanidins detected
Methyl (%) = methylated anthocyanidins (petunidin, peonidin, malvidin) expressed as a percentage of total anthocyanidins detected
Total = the total amounts of anthocyanidins detected (delphinidin, petunidin, malvidin, cyanidin, peonidin, pelargonidin) in mg/g
RHSCC = colors observed described according to the Royal Horticultural Society Color Charts
hue = describes the basic color in degrees as measured by a spectrophotometer with SpectraMagic software (Minolta, Japan)
Ref (%) = describes the percentage of light reflected as measured by a spectrophotometer with SpectraMagic software (Minolta, Japan)

RNA Blot analysis

Flowers of 7 transgenic WKS124/pSPB1532 plants (lines 5-1, 5-2, 7-1, 7-4, 12-1, 12-3) and 7 transgenic Lavande/1532 plants (lines 13-2, 13-3, 13-5, 17-1, 24-2, 34-1) along with flowers from non transgenic WKS124 and Lavande controls were analysed for the presence of transcripts of the introduced *Viola F3'5'H* and *Torenia FMT* transgenes.

Total RNA was isolated from transgenic rose petals with using RNAeasy (Qiagen) following the manufacturer's protocol. Twenty μg of RNA was separated through 1.2 % agarose gel and blotted to Hybond-N (Amersham) following the Instruction Manual of DIG Northern Starter Kit (Roche). RNA probes that hybridized with mRNA of *Viola F3'5'H* (BP#40) and Torenia *FMT* were prepared using the plasmids, pCGP1961 (containing the *Viola F3'5'H* (*BP#40*) cDNA clone) (Australian Provisional Patent Applications No. 2002951088 and 2002952835, 2002, *supra*) and pTMT5 (Figure 8) that had each been digested with the restriction endonuclease *Bam*HI, as the transcription template and the T7 oligonucleotide as the transcription primer following the Instruction Manual of DIG Northern Starter Kit (Roche). Further hybridization and detection were also carried out following the Instruction Manual of DIG Northern Starter Kit (Roche).

Under the conditions used, ~1.7kb trancripts were detected with the *Viola F3'5'H* probe in most of the lines analysed except for line 34-1 (Lavande/pSPB1532). A ~1.0kb transcript was detected with the *TFMT* probe in all 14 transgenic lines analysed. Under the conditions used, no hybridising transcripts were detected in the control petals of WKS124 and Lavande with the *Viola F3'5'H* and *TFMT* probes.

WKS124 transgenic roses

The rose cultivar WKS124 generally produces apricot flowers (RHSCC 38b). HPLC analysis of the anthocyanidins reveal that pelargonidin (0.07mg/g pelargonidin) is the predominant anthocyanidin accumulating with low levels of cyanidin present also (0.01 mg/g cyanidin) (Table 21).

Introduction of the *Viola F3'5'H* chimaeric gene along with the *Torenia FMT* had a dramatic impact on the color of the flowers produced and on the anthocyanidin composition in the petals. In a selection of petals with the most dramatic color change, the 3'5' hydroxylated pigments (delphinidin, petunidin and malvidin) predominated, with malvidin being the most predominant anthocyanin (Table 21).

Introduction of the *Viola F3'5'H* chimaeric gene along with the *Petunia FMT* led to the production of the 3'5' hydroxylated anthocyanidin, delphinidin in a selection of rose petals. The activity of the introduced *Viola* F3'5'H led to the production of relatively high levels of delphinidin (Table 23). However the resulting activity of the introduced *Petunia* FMT in the WKS124 rose petals was low and only a small amount of the methylated anthocyanidin, petunidin accumulated (Table 23). It may be that the physiological conditions within the WKS124 rose petal are not ideal for the *Petunia* FMT to work efficiently.

Production of predominantly delphinidin pigments in a petal background of WKS124 (WKS124/pSPB1534) led to an increase in the total anthocyanidins produced (from 0.08mg/g in the control flowers to 0.5-1.9 mg/g in the transgenic flowers). This production of predominantly delphinidin pigments in WKS124 petals resulted in a change of color from apricot (control flower) to colors in the dark pink to red-purple ranges (Table 23). A similar increase in total anthocyanidins was observed in the transgenic WKS124/pSPB1532 petals (Table 21). However, the delphinidin produced was converted to the methylated petunidin and malvidin-based pigments and this led to a further bluing of flower color into the purple range of colors, resulting in novel colored rose flowers.

The hue values of WKS124/1532 petals are generally closer than those of WKS/1534 petals to 270°, which indicate that malvidin production or methylation of anthocyanins contributes to bluing of flower color. In other words, *FMT* genes are useful to modify flower colors, especially, but not limited, toward blue.

The reflectance values of WKS124/1532 petals are generally lower than those of WKS/1534 petals, which indicate that malvidin production or methylation of anthocyanins

contributes to darkening of flower color. In other words, *FMT* genes are useful to modify flower colors, especially, but not limited, toward darker color. Besides with these flower color changes, WKS124/1532 lines accumulating large amounts of malvidin were more vivid and brilliant in appearance. Such flower color modification is also exhibited by changes of RHSCC. These results clearly demonstrate that *FMT* genes are useful to modify flower color.

Lavande transgenic roses

The rose cultivar Lavande generally produces pink flowers (RHSCC 186c). HPLC analysis of the anthocyanidins reveal that cyanidin (0.08 mg/g cyanidin) is the predominant anthocyanidin accumulating (Table 22).

Introduction of the *Viola F3'5'H* chimaeric gene along with the *Torenia FMT* had a dramatic impact on the color of the transgenic Lavande flowers produced and on the anthocyanidin composition in the petals. In a selection of petals with the most dramatic color change, the 3'5' hydroxylated pigments (delphinidin, petunidin and malvidin) predominated, with malvidin being the most predominant anthocyanin (Table 22).

Introduction of the *Viola F3'5'H* and *Torenia FMT* genes in Lavande led to an increase in the total level of anthocyanidins accumulating in rose petals (from 0.08 mg/g in the control flowers to 0.11-0.36 mg/g in the transgenics) (Table 22).

In this petal background the most dramatic color change and shift to blue (to purple colour 77b) was observed in a flower containing a high proportion (90% of its total anthocyanidins) of delphinidin-based pigments (delphinidin, petunidin and malvidin) with 52% of the total anthocyanidins accumulating being malvidin.

In line 34-1 (Table 22), delphinidin was not produced indicating lack of activity of the introduced *F3'5'H* gene. RNA blot analysis revealed no hybridising *Viola F3'5'H* transcript in this line.. However, a strongly hybridising *Torenia FMT* transcript was detected and the *Torenia* FMT activity was confirmed by the production of peonidin (the methylated

derivative of cyanidin). This result highlighted that the *Torenia* FMT was also able to methylate cyanidin-based pigments.

EXAMPLE 12

Isolation of FMT cDNA clones from Fuchsia spp

PCR of FMT sequences from Fuchsia

CODEHOP design of primers for PCR of FMT sequences from Fuchsia

In order to isolate *FMT* sequences from *Fuchsia*, oligonucleotide primers were designed to areas of amino acid sequence similarity between the *Petunia* FMT (this specification) and published (GenBank database) caffeoyl CoA OMTs (*V. vinifera* (Z54233), *S. longipes* (L22203), *P. tremuloides* (U27116), *P. kitakamiensis* (AB00048), *P. crispum* (Z54183), *E. gunnii* (Y12228), *N. tabacum* (U38612), *M. crystallinum* (AF053553), *A. thaliana* (L40031)).

The CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primers) strategy (Rose *et al.*, *Nucl Acids Res*, *26*: 1628-1635, 1998) (outlined at http://blocks.fhcrc.org/codehop.html) was used. The CODEHOP program designs a pool of primers containing all possible 11- or 12-mers for a 3' degenerate "core" region and having the most probable nucleotide predicted for each position in a 5' non-degenerate "clamp" region (Table 24).

TABLE 24

Oligonucleotides designed to areas of sequence similarity between methyltransferase sequences identified by the CODEHOP program

SEQ ID NO:	PRIMER	SEQUENCE (5' TO 3')
15	OMTIf2	ACC ATC GAG ATC GGC GTN TTY CAN GG
16	OMTIf4	CGA CTT CGC CTT CGT GGA YGC NGA YAA
17	OMTIr3	TGA AGT TGA TCT TGT GCT CCA CNC CNG CYT T
18	OMTIr5	CGC CGG CAG AAG GTG ANN CCR TCN CC

where R = A or G, Y = C or T, M =A or C, K = G or T, S = G or C, W = A or T, H = A or C or T, B = G or C or T, V = A or G or C, D = A or G or T, N = A or G or C or T, I = deoxyinosine.

TABLE 25

Other oligonucleotides designed for use in PCR of FMT sequences

SEQ ID NO:	PRIMER	SEQUENCE (5' TO 3')
19	dT(17)Ad2Ad1	CTG AGA GAA CTA GTC TCG AGC TCT AGA ACA AGC TTT TTT TTT TTT
20	GI-anchor	GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG
27	Ad1	CTG AGA GAA CTA GTC TCG AG

I = deoxyinosine

Total RNA was isolated from *Fuchsia* petal buds using the Plant RNAeasy kit (QIAGEN). One microgram of RNA was used as a template to synthesize cDNA using Superscript II (Stratagene) and the dT(17)Ad2Ad1 [SEQ ID NO: 19] (Table 25) oligonucleotide under conditions as recommended by the manufacturer. The cDNA was purified by passing it through a PCR purification column (QIAGEN) and eluting in 50 µL 10 Mm Tris-HCl, pH 8.5. The cDNA was subsequently C-tailed using Calf Thymus terminal transferase

(Boehringer Mannheim) using conditions recommended by the manufacturer. The C-tailed cDNA was then purified through a PCR purification column (QIAGEN) and eluted in 50 µL 10 mM Tris-HCl, pH8.5.

The C-tailed cDNA (1 µL) was subsequently used as template in a PCR with 2.5 µL 10 x HotSTAR (trademark) Taq QIAGEN buffer, 4 µL 1.25 mM dNTP, 5 µL 50 ng/µL primer OMTIf2 [SEQ ID NO: 15], 5 µL 50 ng/µL Ad1 primer [SEQ ID NO: 27] (Table 25), 2 µL pure water and 0.5 µL HotSTAR (trademark) Taq DNA polymerase (QIAGEN). The reaction was heated to 95°C for 15 minutes then run through 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 90 seconds, followed by 72°C for 10 minutes.

The PCR products were electrophoresed through a 1% w/v agarose gel and expected products of around 0.8 kb in length were excised, purified and ligated with pCR 2.1 (Invitrogen). A random selection of transformants was analysed for the presence of inserts by digesting with the restriction endonuclease *Eco*RI. Transformants containing inserts of 0.8 kb were sequenced using the M13 Reverse and M13 Forward -21 primers. An example of resulting *Fuchsia* sequences showing similarity to FMTs is found in the plasmid designated pCGP3267 (Figure 21).

The *Fuchsia FMT* (SEQ ID NO: 21) contained in pCGP3267 showed 66% and 64% identity at the nucleotide level with the *Petunia* [SEQ ID NO:4] and *Torenia FMT*]SEQ ID NO:11] when comparing the coding sequence corresponding only with the length of the partial *Fuchsia FMT* clone. The deduced amino acid sequence of encoded by the *Fuchsia FMT* clone in pCGP3267 showed 81% similarity with both the *Petunia* [SEQ ID NO:5] and *Torenia FMT* [SEQ ID NO:12] again considering only the region comparable to the length of the partial *Fuchsia* clone.

Generation of full-length Fuchsia FMT clone

A genomic strategy was employed to generate sequence upstream of the *Fuchsia FMT* cDNA clone [SEQ ID NO: 21] contained in the plasmid pCGP3267 (Figure 21).

Isolation of genomic DNA from Fuchsia

Plasmid genomic library construction

Genomic DNA (gDNA) was extracted from 1 g of fresh, young leaf material of *Fuchsia hybrida* cultivar Derby Imp using the Qiagen DNeasy maxi kit and following the manufacturers instructions. Approximately 1.2 µg of gDNA was then digested with the restriction endonuclease, *Taq*I. The digested genomic DNA fragments were then ligated (using Amersham ligation kit) with dephosphorylated *Eco*RV ends of the vector pBluescript II (Stratagene). The ligation mix was then used as a template in PCR.

The primer OMTIf1 [SEQ ID NO: 23] along with the primer FucR1 [SEQ ID NO: 34] (Table 26) that was designed to the Fuchsia *FMT* cDNA clone contained in pCGP3267 were used in a PCR using *Fuchsia* genomic DNA as template. The amplified products were purified and ligated into the vector pCR2.1. Sequence analysis of a 274 bp fragment (designated as the "OMTIf1/FucR1 amplified fragment") revealed that this fragment included 51 bp of overlapping sequence with the *Fuchsia FMT* cDNA clone in the plasmid pCGP3267, a further 74 bp of new coding sequence upstream of this point, an intron that was 88 bp in length and a further 61 bp of new coding sequence upstream from the intron.

Further to this a nested primer pair combination (FucR5 [SEQ ID NO:36] and FucR6 [SEQ ID NO:37] was then designed to the sequence that was upstream from the intron. The primers FucR5 [SEQ ID NO:36] and FucR6 [SEQ ID NO:37] were used on *Fuchsia* gDNA that had been digested with the restriction endonuclease, *Taq*I. The products that were amplified were ligated with the *Acc*I ends of the vector pBluescript KS (Stratagene). A first round of amplification by PCR was performed using the primersFucR5 [SEQ ID NO:36] and M13rev (NEB) and *Fuchsia* gDNA as template. The products were purified using a Qiaquick column (QIAGEN) and then added as template to the second round of PCR amplification with the primers FucR6 [SEQ ID NO:36] and T3 (Stratagene). The amplified products were purified and ligated into the vector pCR2.1. Sequence analysis of

a 247 bp fragment (designated "FucR6/T3 amplified fragment") revealed a further 24 bp of new coding sequence upstream of that obtained with the "OMTIf1/FucR1 amplified fragment". The remainder of the sequence consisted of another intron that was 223 bp in length and no further coding sequence could be identified upstream of this. A further 51 to 54 bp of sequence (i.e. 17 or 18 amino acids) were required to reach the presumed methionine start as determined by comparison with the *Torenia* and *Petunia FMT* sequences. Therefore, a strategy was developed to utilize the 5' sequence of the *Torenia FMT* cDNA clone and ligate this with the longest *Fuchsia FMT* PCR product to generate a full-length and functional *Fuchsia FMT* cDNA clone.

A primer (FucF1) [SEQ ID NO:38] was designed to the 5'end of the coding sequence found in the FucR6/T3 amplified fragment (described above). The FucF1 primer [SEQ ID NO:38] and the Ad1 primer [SEQ ID NO:27] were used in a PCR with *Fuchsia* cDNA as template (synthesis of *Fuchsia* cDNA described above). The amplified product was cloned into pCR2.1 and the resulting plasmid was designated pCGP3282. The plasmid pCGP3282 was used as template in a PCR with the Ad1 [SEQ ID NO:27] and Tor-5'pos [SEQ ID NO: 39] primers and Taq DNA polymerase HotSTAR taq (QIAGEN). The use of the Taq DNA polymerase, HotSTAR taq (QIAGEN) leaves a 3'-A overhang on the amplified product. The resulting amplified product (defined as "Tor-5' pos/Ad1 amplified fragment") was then digested with the restriction endonuclease, *Spe*I. (an *Spe*I recogintion sequence is located within the Ad1 primer at the 3' end of the cDNA clone).

The primers Tor-5'pos [SEQ ID NO:39] and Tor-5'neg [SEQ ID NO:40] were annealed together upon incubation at 75°C for 5 minutes, followed by a slow cooling down to 37°C over 30 minutes . These primers were designed so that once annealed there would be a "T" overhang at the 3' end of the sequence. and sequence overhang compatible with an EcoRI recogntion sequence at the 5'-end. The annealed oligonucleotide were ligated with the *Spe*I ends of the "Tor-5' pos/Ad1 amplified fragment" . This ligated products were then used as template in a PCR using the oligonucleotides Tor-5'pos [SEQ ID NO:39] and Ad1

[SEQ ID NO:27] as primers. The PCR product was then ligated with the cloning vector pCR2.1. The resulting plasmid was designated pCGP3289 (Figure 22).

The *Fuchsia FMT* [SEQ ID NO: 43] contained in pCGP3289 showed 51%, 48% and 56% identity at the nucleotide level with the *Petunia E20* [SEQ ID NO:4], *Petunia E33* [SEQ ID NO:26] and *Torenia FMT* [SEQ ID NO:11], respectively. The deduced amino acid sequence encoded by the *Fuchsia FMT* clone in pCGP3289 [SEQ ID NO:44] showed 67%, 80% and 82% similarity with the *Petunia* E20 [SEQ ID NO:5], *Petunia E33* [SEQ ID NO:7] and *Torenia FMT* [SEQ ID NO:12], respectively.

TABLE 26

Primers

SEQ ID NO:	NAME	SEQUENCE 5' TO 3'
34	FucR1	GCA AGT GCA GTG CAA AGA AGA G
35	FucR3	GAT CTT ATG TTC CAC TCC GC
36	FucR5	GAG AGA TCT GAC CAG TAA GG
37	FucR6	GGA TAT TTT TCG GCC GTG ACC TCC
38	FucF1	ATC TTA GAG ACG ACT GCT TAT CCC
39	Tor-5'pos	AAT TCG CAG CAA AAA TGA AAG ATA AGT TCT ATG GCA CCA TTT TGC AGA GCG AAG CCC TCG CAA AGT AT
40	Tor-5'neg	TAC TTT GCG AGG GCT TCG CTC TGC AAA ATG GTG CCA TAG AAC TTA TCT TTC ATT TTT GCT GCG

Construction of pCGP3292 (35S 5': FFMT: 35S 3'; 35S 5': Viola F3'5'H: 35S 3'; 35S 5': SuRB binary vector)

The binary plasmid pCGP3292 (Figure 25) was constructed to allow the production of methylated delphinidin derivatives such as petunidin and malvidin in a line that does not normally produce delphinidin-based pigments and does not contain a flavonoid methyltransferase capable of methylating delphinidin-based anthocyanins.

The binary plasmid pCGP3292 (Figure 25) contains a *35S 5': FFMT: 35S 3'* expression cassette (from the plasmid pCGP3290 (Figure 23)) and a *35S 5': Viola F3'5'H: 35 3'* expression cassette, both in tandem with the *35S 5': SuRB* selectable marker cassette of the Ti binary vector of pCGP1988 (Figure 12).

Construction of intermediate plasmids

(i) Construction of pCGP3290 (35S 5': FFMT: 35S 3' expression cassette)

The plasmid pCGP3290 (Figure 23) was constructed by cloning the *Fuchsia FMT* (*FFMT*) cDNA clone from pCGP3289 (Figure 22) into a *CaMV 35S* expression cassette.

The plasmid pRTppoptcAFP was used as a source of a *CaMV 35S* promoter and terminator fragments. It was initially digested with the restriction endonuclease *Xba*I, the overhanging 5' ends were repaired and then the plasmid was digested with the restriction endonuclease *Eco*RI to release the 3.3kb vector containing the *CaMV 35S* expression cassette. The 3.3 kb fragment was isolated and purified.

The plasmid pCGP3289 (Figure 22) was digested initially with the restriction endonuclease *Spe*I and the resulting 5' overhang ends were repaired. The linearized plasmid was then restricted with the restriction endonuclease *Eco*RI to release a 1.0 kb *Fuchsia FMT* cDNA fragment which was isolated, purified and then ligated with the *Xba*I (blunt)/*Eco*RI ends of the pRTppoptc vector (described above). Correct ligation of the fragments was established by restriction endonuclease analysis (*Hin*DIII, *Xho*I, and *Pst*I,) of plasmid DNA isolated from ampicillin-resistant transformants. The resulting plasmid was designated pCGP3290 (Figure 23).

(ii) Construction of pCGP2788 (35S 5': Viola F3'5'H: 35S 3'; 35S 5': SuRB binary vector)

The binary plasmid pCGP2788 (Figure 24) contains the 35S 5': Viola F3'5'H: 35S 3' expression cassette (from pCGP3254 (Figure 13) in tandem with the *35S 5': SuRB* selectable marker cassette of the Ti binary plasmid pCGP1988 (Figure 12).

The binary plasmid pCGP3254 (Figure 13) was digested with the restriction endonuclease *PstI* to release the *35S 5': Torenia FMT: 35S 3'* expression cassette and the expression binary vector backbone. The resulting fragments were ethanol precipitated (Sambrook *et al.*, 1989, *supra*) and the mixture of fragments was religated. Correct ligation of the vector backbone containing the *35S 5': SuRB* gene and the chimaeric *Viola F3'5'H* gene without the *35S 5': Torenia FMT: 35S 3'* cassette was established by restriction endonuclease analysis (*Hin*DIII, *EcoRV*, *Pst*I, *Eco*RI, *and NcoI*) of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP2788 (Figure 24).

Construction of pCGP3292 (35S 5': FFMT: 35S 3'; 35S 5': Viola F3'5'H: 35S 3'; 35S 5': SuRB expression binary)

Plasmid pCGP3292 (Figure 25) was constructed by cloning the chimaeric *Fuchsia FMT* gene from pCGP3290 (Figure 23) into the Ti binary vector pCGP2788 (Figure 24).

The *35S 5': FFMT: 35S 3'* expression cassette from pCGP3290 (Figure 23) was released upon digestion with the restriction endonuclease *Pst*I. A 1.66 kb fragment containing the chimaeric *Fuchsia FMT* gene was subsequently isolated and ligated with *Pst*I ends of the binary vector, pCGP2788 (Figure 24). Correct ligation of the chimaeric gene in tandem with the *35S 5': SuRB* gene and the chimaeric *F3'5'H* gene of pCGP2788 was established by restriction endonuclease analysis (*Hin*DIII, *Xho*I, *Pst*I, *Eco*RI, *and NcoI*) of plasmid DNA isolated from tetracycline-resistant transformants. The resulting plasmid was designated pCGP3292 (Figure 25).

Plant transformation with pCGP3292

The binary vector plasmid pCGP3292 was introduced into *A. tumefaciens* strain AGL0 and the T-DNA contained in pCGP3292 is introduced into *Rosa hybrida via Agrobacterium*-mediated transformation to produce petunidin and malvidin based pigments and lead to modifications flower colour (as detailed in Example 11)

EXAMPLE 13

Dendogram of plant methyltransferases

A dendogram was constructed using the software package ClustalW (Thompson *et al.*, 1994, *supra*) (Figure 26). Deduced amino acid sequences of Petunia (pCGP1907.aa), *Torenia* (pTMT5.aa) and *Fuchsia* (pCGP3267.aa) *FMTs* were aligned with other full length plant *O*-methyltransferases of both Class I and Class II found in the GenBank database. The dendogram (Figure 26) shows the clustering relationship between these sequences. All Class I SAM-OMT sequences are grouped together due to their overall level of sequence similarity. The Petunia, *Torenia* and *Fuchsia* FMT sequences are grouped with Class I SAM-OMTs. However, they are set apart from the main cluster. This indicates that these sequences are related to each other but share a lower level of sequence identity and similarity with other SAM-OMTs within this class. All other Class I SAM-OMTs have been identified as CCoAOMTs either by testing for corresponding enzyme activity with CoA-activated phenylpropanoid substrates derived from caffeic acid, or by sequence similarity with database entries. The sequences of an *A. thaliana* CCoAOMT (GenBank L40031) and a *Populus kitakamiensis* CCoAOMT (GenBank AB000408) are found in an adjacent cluster to that of the FMTs described here. These sequences are more similar to the FMTs than other CCoAOMTs. However, no experimental evidence exists for these clones regarding enzyme activity or substrates that are methylated. The remaining branches of the dendogram are formed by groupings of Class II SAM-OMTs. These include COMTs (caffeic acid OMTs), F3'OMT (flavonoid 3'-OMT; Gauthier *et al.*, 1996, *supra*), IOMTs (isoflavone OMTs; He and Dixon, 1998, *supra*), 2'OMTs (isoliquiritigenin 2'-OMT; Maxwell *et al.*, 1993, *supra*), IMT (inositol OMT; Rammesmeyer *et al.*, 1995,

supra), and F7OMT (flavonoid 7-OMT; Christensen *et al.*, 1998, *supra*), among others. Given the variety of substrates utilized by the members of Class II SAM-OMTs, and the ability of some of these proteins to act on flavonoid compounds which are structurally related to the anthocyanins, it was unexpected that the FMTs isolated from *Petunia*, *Torenia* and *Fuchsia* do not fall into this category of SAM-OMTs. Reviews in the literature (Ibrahim and Muzac, 2000, *supra*; Schroder *et al., Phtochemistry, 59:* 1-8, 2002) have suggested that methyltransferases acting on flavonoids and specifically on anthocyanins would fall into the Class II SAM-OMTs. Surprisingly the FMT sequences disclosed in this specification resemble the CCoAOMTs in Class I more closely than members of the Class II SAM-OMTs. CCoAOMTs are known to efficiently utilize only a pair of CoA-activated substrates, caffeoyl-CoA (CCoA) and 5-hydroxyferuloyl-CoA (HFCoA). These phenylpropanoid compounds are directly derived from caffeic acid (CA) and 5-hydroxyferulic acid (HFA) which are efficiently utilized by COMT proteins of Class II SAM-OMTs. The basic ring structure of these flavonoids and anthocyanins is similar, the main difference with the anthocyanins being the presence of sugar and acyl side groups which form bulky additions to the molecule. It is thought that these groups may impose different steric requirements upon enzymes involved in modification of anthocyanins compared with, for example, flavanone and isoflavonoid molecules. Thus, in regard to anthocyanin compounds, the sugar and acyl side groups may mimic the large CoA group attached to these molecules imposing a similar steric requirement on SAM-OMT proteins that act on them.

EXAMPLE 14

Isolation of FMT cDNAs from other species

Methylated anthocyanins such as but not limited to peonidin, petunidin and malvidin are produced in *Petunia sp.*, *Plumbago sp.*, *Vitis sp.*, *Babiana stricta*, *Pinus sp.*, *Picea sp.*, *Larix sp.*, *Phaseolus sp.*, *Solanum sp.*, *Vaccinium sp.*, *Cyclamen sp.*, *Iris sp.*, *Pelargonium* sp., *Geranium sp.*, *Pisum sp.*, *Lathyrus sp.*, *Clitoria sp.*, *Catharanthus sp.*, *Malvia sp.*, *Mucuna sp.*, *Vicia sp.*, *Saintpaulia sp.*, *Lagerstroemia sp.*, *Tibouchina sp.*, *Hypocalyptus sp.*, *Rhododendron sp.*, *Linum sp.*, *Macroptilium sp.*, *Hibiscus sp.*, *Hydrangea sp.*, *Ipomoea*

sp., *Cymbidium sp.*, *Millettia sp.*, *Hedysarum sp.*, *Lespedeza sp.*, *Antigonon sp.*, *Pisum sp.*, etc..

It is expected that a number of these plants contain flavonoid methyltransferases (FMT).

Rare methylated anthocyanins (such as 5-methyl delphinidin, 5-methyl petunidin and 5-methyl malvidin) have been isolated from flowers of plants in the Plumbaginaceae family Harborne, 1967, *supra*). *Plumbago* flowers have been reported to contain a rare anthocyanin that is methylated at the 5-*O* position of malvidin. This molecule was described as capensinin (5-*O*-methyl malvidin) (Harborne, 1962, 1967, *supra*). The flavonol copigment present was described as azalein (quercetin 5-methyl ether 3-*O*-rhamnoside) (Harborne, 1962, 1967, *supra*). Further analysis of the common garden *Plumbago capsensis* (also known as *Plumbago auriculata*) has revealed that the methylated anthocyanin was 5, 7-di-*O*-methyl malvidin (S. Bloor, unpublished results). It is expected that flowers from plants in the Plumbaginaceae family such as *Plumbago* are a suitable source for *FMT* sequences that encode FMTs that would methylate anthocyanins at positions 3', 5', 3' and 5' as well as the 5-*O* and 7-*O* positions.

The isolation of *FMT* cDNAs from the plants listed above and others is accomplished by the screening of respective cDNA libraries with SEQ ID NO:1 and/or4 and/or 6 and/or 11 and/or 21 and/or 26 and/or 41, and/or 43 using low stringency hybridisation conditions such as those described Example 9 or in the introduction of the instant specification.

Alternatively, the isolation of *FMT* cDNA fragments are accomplished using the polymerase chain reaction using CODEHOP primers as listed in Table 24 (Example 11) or degenerate primers as listed in Table 27, below. An example of the primer pair combinations that can be used is shown in Table 28, below. The amplification products are cloned into bacterial plasmid vectors and DNA fragments used as probes to screen respective cDNA libraries to isolate longer and full-length *FMT* cDNA clones. The

functionality and specificity of the cDNA clones are ascertained using methods described in Examples 7, 8, 9, 10 and 11.

TABLE 27

More degenerate primers designed to areas of amino acid sequence similarity between methyltransferases that act on anthocyanins

SED ID NO:	PRIMER	SEQUENCE (5' TO 3')
23	OMTIf1	CCG GGA GCA CGA GCA CYT NAA RGA RYT
24	OMTIf3	GGC CTG CCC TTC ATC CAR AAR GCN GGN G
25	OMTIr4	CGT GGT AGT TCA CGT AGT TGC TCT TRT CNG CRT C

where R = A or G, Y = C or T, M =A or C, K = G or T, S = G or C, W = A or T, H = A or C or T, B = G or C or T, V = A or G or C, D = A or G or T, N = A or G or C or T, I = deoxyinosine.

TABLE 28

Primer pairs that are used in the isolation of other FMT cDNA fragments from different plants

Forward primer	SEQ ID NO:	Reverse primer	SEQ ID NO:	Expected fragment (bp)
OMTIf1	21	OMTIr3	17	285
OMTIf1	21	OMTIr4	23	399
OMTIf1	21	OMTIr5	18	609
OMTIf1	21	Ad1	27	620 + 3'UTR
OMTIf2	15	OMTIr3	17	159
OMTIf2	15	OMTIr4	23	273
OMTIf2	15	OMTIr5	18	483
OMTIf2	15	Ad1	27	492 + 3'UTR

Forward primer	SEQ ID NO:	Reverse primer	SEQ ID NO:	Expected fragment (bp)
OMTIf3	22	OMTIr4	23	162
OMTIf3	22	OMTIr5	18	372
OMTIf3	22	Ad1	27	381 + 3'UTR
OMTIf4	16	OMTIr5	18	258
OMTIf4	16	Ad1	27	267 + 3'UTR
OMTIr3	17	GI-anchor	20	375 + 5'UTR
OMTIr4	23	GI-anchor	20	489 + 5'UTR
OMTIr5	18	GI-anchor	20	699 + 5'UTR

+ 3'UTR = plus the 3' untranslated sequence, + 5'UTR = plus the 5' untranslated sequence

Estimations of the expected size of fragment are based on the *Petunia FMT* (*E20*) sequence [SEQ ID NO:4]. The sizes obtained using cDNA as template from different species would be expected to vary.

EXAMPLE 15

Use of FMTs

In order to produce methylated dephinidin pigments in plants that do not normally produce delphinidin-based pigments and does not contain a flavonoid methyltransferase capable of methylating anthocyanidins, specifically delphinidin, constructs containing the combination of a *F3'5'H* gene (such as but not limited to the chimaeric *Viola F3'5'H* gene) and a *FMT* gene (such as but not limited to those isolated from *Petunia*, *Fuchsia*, *Torenia*, *Plumbago*) are introduced into a species that does not normally produce delphinidin-based pigments. Such plants may include but are not limited to carnation, chrysanthemum, gerbera, orchids, *Euphorbia, Begonia* and *apple.*

In order to produce methylated pigments in species or cultivars of species that produce delphinidin or cyanidin but do not have a flavonoid methyltransferase capable of methylating these anthocyanins, *FMT* genes are introduced into plant species or specific

cultivars of species that do not produce methylated anthocyanin pigments. Such plants include but are not limited to pansy, *Nierembergia*, lisianthus, cultivars of grapevine and lily.

In order to reduce or block the production of indigenous methylated pigments a variety of strategies can be employed including but not limited to PTGS, RNAi, antisense, co-suppression technologies. Strategies include the introduction of *FMT* sequences into plant species or cultivars of species thar produce methylated anthocyanin pigments such as petunidin, malvidin, peonidin, capsenidin or other methylated anthocyanin. Such species include those described in Example 14, such as *Impatiens*, *Catharanthus*, cyclamen, *Torenia*, *Petunia*, *Fuchsia*, *Plumbago*, *Pelargonium* and certain cultivars of grapevine.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

BIBLIOGRAPHY

Altschul *et al.*, *Nucl. Acids Res. 25:* 3389, 1997

Altschul *et al.*, *J. Mol. Biol. 215:* 403-410, 1990

Andersen *et al.*, *Pytochemistry, 38*: 1513-1517, 1995

Andersen, *Biochemical systematics and ecology, 20*: 145-148, 1992

Ando *et al.*, *Biochemical systematics and ecology, 27*: 623-650, 1999

Australian Provisional Patent Applications No. 2002951088 and 2002952835 entitled "Genetic Sequences and uses therefor", 2002

Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons Inc, 1994-1998, Chapter 15, 1998

Aviv and Leder, *Proc. Natl. Acad. Sci. USA 69*: 1408, 1972

Ballington *et al.*, *Can. J. of Plant Sci., 68*: 241-246, 1988

Bonner and Laskey, *Eur. J. Biochem. 46*: 83, 1974

Brouillard and Dangles, In: *The Flavonoids - Advances in Research since 1986*. Harborne, J.B. (ed), Chapman and Hall, London, UK, 1-22, 1993

Brugliera *et al.*, *Plant J. 5*, 81-92, 1994

Bullock *et al.*, *Biotechniques 5*: 376, 1987

Cachio *et al.*, *American J of Ecology and Viticulture, 43*: 244-248, 1992

Carew and Krueger, *Phytochemistry, 15:* 442, 1976

Catalano *et al.*, *J. Agricultural and Food Chemistry, 49*: 4568-4570, 1998

Chriki and Harborne, *Phytochemistry, 22*: 2322-2323, 1983

Chriki, *Agronomie, 10*: 553-540, 1990

Christensen *et al.*, *Plant Mol. Biol. 36*: 219-227, 1998

Comai *et al.*, *Plant Mol. Biol.* 15: 373-381, 1990

Comai *et al.*, *Plant Molecular Biology 15:* 373-381, 1990

Crowden, *Phytochemistry, 21*: 2989-2990, 1982

Depicker, A. *et al.*, *J Mol. and Appl. Genetics,* 1: 561-573, 1982

Francis *et al.*, *J Am Soc Hortic Sci, 107*: 789-791, 1982

Franck *et al.*, *Cell* 21: 285-294, 1980

Fukui *et al.*, *Phytochemistry, 47:* 1409-1416, 1998

Gamborg *et al.*, *Exp. Cell Res. 50*: 151-158, 1968

Gauthier *et al.*, *Plant Mol. Biol. 32:* 1163-1169, 1996

Goto, *Tetrahedron 27:* 2413-2416, 1987

Griesbach *et al.*, *Phytochemistry 30*: 1729-1731, 1991

Griesbach, *Phytochemistry, 48*: 829-830, 1998

Guilley *et al.*, *Cell*, 30: 763-773. 1982

Hanahan, *J. Mol. Biol. 166*: 557, 1983 and. *Bethesda Res. Lab. Focus. 8(2)*: 9, 1986

Haslam, *Practical Phenolics. From structure to molecular recognition and physiological action*. Cambridge University Press, UK, 1998

He and Dixon, *Plant Mol. Biol. 36*: 43-54, 1998

Holton and Cornish, *Plant Cell 7*: 1071-1083, 1995;

Holton *et al.*, *Nature, 366*: 276-279, 1993

Hopp and Seitz, *Planta 170*: 74-85, 1987

Hungria *et al.*, *Plant Physiology, 97*: 751-758, 1991

Ibrahim and Muzac, In *Recent advances of phytochemistry.* Evolution of metabolic pathways. Elsevier Science Ltd. *34*: 349- 385, 2000

Imrie and Hutton, *J. Hered., 69*: 54-56 1978

Inoue *et al.*, *Gene 96*: 23-28, 1990

Inoue *et al.*, 1990

Ishikura and Shibata, *Bot Mag (Tokyo), 86:* 1-4, 1973

Jefferson *et al.*, *EMBO J. 6(13)*: 3901-3907, 1987

Jefferson, *et al.*, *EMBO J.* 6: 3901-3907, 1987

Jonsson *et al.*, *Phytochemistry 21(10)*: 2457-2460, 1982

Jonsson *et al.*, *Planta 160:* 174-179, 1984a

Jonsson *et al.*, *Theor. Appl. Genet. 66:* 349-355, 1983

Jonsson *et al., Theor. Appl. Genet. 68:* 459-466, 1984b

Joshi and Chiang, *Plant Mol. Biol. 37*: 663-674, 1998

Kim and Fujieda, *J. Kor. Soc. Hortic. Sci., 32*: 247-255, 1991

Kim *et al.*, *Phytochemistry, 28*: 1503-1506, 1989

Klee *et al.*, *Bio/Technology 3:* 637-642, 1985

Kobayashi *et al.*, *Breeding Science, 48*: 169-176, 1998

Kroon *et al.*, *Plant J 5*: 69-80, 1994

Lazo *et al.*, *Bio/technology 9*: 963-967, 1991

Lee *et al.*, *EMBO J.* 7: 1241-1248, 1988

Lewis *et al., J. of the Science of Food and Agriculture, 77*: 45-57, 1998

Marchant *et al., Molecular Breeding 4*: 187-194, 1998

Markham, *Techniques of flavonoid identification.*, Academic Press, London, 1982

Marmur and Doty, *J. Mol. Biol. 5*:109, 1962

Maxwell, *Plant J. 4(6)*: 971-981, 1993

Merrifield, *J. Am. Chem. Soc. 85:* 2149, 1964

Mikanagi *et al.*, *Biochem. System and Ecol. 23:* 183-200, 1995

Mikanagi *et al.*, *Biochem. System and Ecol. 28*: 887-902, 2000

Mitchell *et al.*, *Phytochemistry, 47*: 355-361, 1998

Mitsuhashi *et al., Plant Cell Physiol. 37*: 49-59, 1996

Mol *et al.*, *Trends Plant Sci. 3:* 212–217, 1998

Murashige and Skoog, *Physiol. Plant 15*: 73-97, 1962

Nozzolillo *et al.*, *Canadian Journal of Botany, 67*: 1600-1604, 1989

Parvez and Ogbeide, *Phytochemistry, 29*: 2043-2044, 1990

Pearson and Lipman, *Proc. Natl. Acad. Sci. USA 85*: 2444-2448, 1988

Rammesmeyer *et al.*, *Arch. Biochem. Biophys. 322(1)*: 183-188, 1995

Rat'kin *et al.*, *Zh Obshch Biol, 41*: 685-699, 1980

Robinson and Firoozabady, *Scientia Horticulturae, 55*: 83-99, 1993

Rose *et al.*, *Nucl Acids Res*, *26*: 1628-1635, 1998

Rout *et al.*, *Scientia Horticulturae*, *81*: 201-238, 1999

Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual.* (2nd edition), Cold Spring Harbor Laboratory Press, USA, 1989

Schenk and Hildebrandt, *Can. J. Bot. 50*: 199-204, 1972

Schroder *et al., Phtochemistry, 59:* 1-8, 2002

Seitz and Hinderer, Anthocyanins. In: *Cell Culture and Somatic Cell Genetics of Plants.* Constabel, F. and Vasil, I.K. (eds.), Academic Press, New York, USA, *5*: 49-76, 1988

Sink (ed), *Petunia,* Springer-Verlag, Berlin, 1984

Skrede *et al.*, *J of Food Science*, *65*: 357-364, 2000

Srivastava and Pande, *Planta Med, 32*: 138-140, 1977

Stafford, *Flavonoid Metabolism.* CRC Press, Inc. Boca Raton, Florida, USA, 1990

Strack and Wray, In: *The Flavonoids -Advances in Research since 1986.* Harborne, J.B. (ed), Chapman and Hall, London, UK, 1-22, 1993

Takeda *et al.*, *Phytochemistry*, 28: 499-500, 1989

Takeda *et al.*, *Phytochemistry*, *29*: 1089-1091, 1990

Takeoka *et al.*, *Journal of Agricultural and Food Chemistry*, *45*: 3395-3400, 1997

Tanaka *et al.*, *Plant Cell Physiol 37*: 711-716, 1996

Terahara *et al.*, *J. Natural Products*, *56*: 335-340, 1993

Thompson *et al.*, *Nucl. Acids Res. 2*: 4673-4680, 1994

Tiwari and Minocha, *Vijnana Parishad Anusandhan Patrika, 23*: 305-308, 1980

Toki and Katsuyama, *J. Jap Soc Hortic. Sci.*, *63*: 853-861, 1995

Toki *et al.*, *Phytochemistry, 37*: 885-887, 1994

Turpen and Griffith, *BioTechniques 4:* 11-15, 1986

Van Wyk *et al.*, *Biochemical systematics and ecology, 23*: 295-297, 1995

VanEngelen *et al.*, *Transgenic Res.4*: 288-290, 1995

Webby and Boase, *Phytochemistry, 52*: 939-941, 1999

Winkel-Shirley, *Plant Physiol. 126*: 485-493, 2001a

Winkel-Shirley, *Plant Physiol. 127*: 1399-1404, 2001b

Woltering and Somhorst, *J. Plant Physiol.*, *136*: 295-299, 1990

Yabuya and Noda, *Euphytica, 103*: 325-328, 1998

Yabuya *et al.*, *Euphytica, 98*: 163-167, 1997

CLAIMS

1. An isolated nucleic acid comprising a sequence of nucleotides encoding or complementary to a sequence encoding a flavonoid methyltransferase (FMT) or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

2. The isolated nucleic acid molecule of claim 1 wherein the FMT modulates or otherwise facilitates the methylation of an anthocyanin.

3. The isolated nucleic acid molecule of any one of claim 1 or 2 wherein the FMT is a Class I *S*-adenosyl-L-methionine *O*-methyltransferase (SAM-OMTs).

4. The isolated nucleic acid molecule of claim 3 wherein the FMT is 3'FMT or 3'5'FMT.

5. The isolated nucleic acid molecule of claim 2 wherein the anthocyanin molecule is derivative of delphinidin.

6. The isolated nucleic acid molecule of claim 2 wherein the anthocyanin molecule is a derivative of petunidin or cyanidin.

7. The isolated nucleic acid molecule of claim 2 wherein the anthocyanin molecule is delphinidin 3-glucoside, delphinidin 3, 5-diglucoside or delphinidin 3-rutinoside.

8. The isolated nucleic acid molecule of claim 2 wherein methylation of an anthocyanin molecule results in the production of a petunidin, malvidin or peonidin derivative.

9. The isolated nucleic acid molecule of claim 1 wherein the molecule is of *Petunia, Torenia, Fuchsia, Plumbago* or botanically related plant origin.

10. An isolated nucleic acid molecule according to any one of claims 1 to 3 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:1;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:1;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 1 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:2;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:2;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent or homolog thereof.

11. The isolated nucleic acid molecule of any one of claims 1 to 3 having the nucleotide sequence comprising:-

(i) a nucleotide sequence set forth in SEQ ID NO:4;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:4;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 4 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:5;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:5;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

12. The isolated nucleic acid molecule of any one of claims 1 to 3 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:6;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:6;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO:6 or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

13. An isolated nucleic acid molecule of any one of claims 1 to 3 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:26;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:26;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 26 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:7;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:7;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

14. An isolated nucleic acid molecule of any one of claims 1 to 3 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:11;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:11;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 11 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:12;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:12;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

15. An isolated nucleic acid molecule of any one of claims 1 to 3 having the nucleotide sequence comprising:-

(i) a nucleotide sequence set forth in SEQ ID NO:21;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:21;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 21 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:22;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:22;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

16. An isolated nucleic acid molecule of any one of claims 1 to 3 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:41;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:41;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 41 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:42;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:42;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

17. An isolated nucleic acid molecule according to any one of claims 1 to 3 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:43;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:43;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 43 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:44;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:44;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

18. A genetic construct comprising a nucleic acid molecule encoding or complementary to a sequence encoding an FMT of any one of claims 1 to 3 or 9 or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent or homolog thereof.

19. The genetic construct according to claim 18 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:1;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:1;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 1 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:2;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:2;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

20. The genetic construct of claim 18 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:4;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:4;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 4 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:5;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:5;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

21. The genetic construct of claim 18 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:6;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:6;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 6 or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

22. The genetic construct of claim 18 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:26;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:26;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 26 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:7;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:7;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

23. The genetic construct of claim 18 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:11;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:11;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 11 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:12;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:12;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

24. The genetic construct of claim 18 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:21;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:21;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 21 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:22;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:22;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

25. The genetic construct of claim 18 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:41;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:41;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 41 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:42;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:42;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

26. The genetic construct of claim 18 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:43;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:43;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 43 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:44;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:44;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

27. A genetically modified plant or part thereof or cells therefrom comprising genetic material encoding or complementary to a sequence encoding an FMT of any one or

claims 1 to 3 or 9 or a mutant, part, derivative, fragment or portion thereof or a functional and/or structural equivalent or homolog or analogue.

28. The genetically modified plant or part thereof or cells therefrom of claim 27 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:1;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:1;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 1 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:2;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:2;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

29. The genetically modified plant or part thereof or cells therefrom of claim 27 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:4;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:4;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 4 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:5;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:5;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

30. The genetically modified plant or part thereof or cells therefrom of claim 27 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:6;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:6;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 6 or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

31. The genetically modified plant or part thereof or cells therefrom of claim 27 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:26;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:26;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 26 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:7;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:7;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

32. The genetically modified plant or part thereof or cells therefrom of claim 27 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:11;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:11;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 11 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:12;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:12;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

33. The genetically modified plant or part thereof or cells therefrom of claim 27 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:21;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:21;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 21 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:22;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:22;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

34. The genetically modified plant or part thereof or cells therefrom of claim 27 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:41;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:41;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 41 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:42;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:42;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

35. The genetically modified plant or part thereof or cells therefrom of claim 27 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:43;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:43;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 43 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:44;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:44;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

36. The genetically modified plant or part thereof or cells therefrom of claim 27 wherein said plant or part thereof or cells therefrom is from a cut-flower species.

37. The genetically modified plant or part thereof or cells therefrom of claim 27 wherein said plant or part thereof or cells therefrom is a horticultural plant species.

38. The genetically modified plant or part thereof or cells therefrom of claim 27 wherein said plant or part thereof or cells therefrom is an agricultural plant species.

39. The genetically modified plant or part thereof or cells thereof of claims 37 or 38 wherein the plant exhibits altered flowers or inflorescence.

40. The genetically modified plant or part thereof or cells therefrom of claim 36 or 37 or 38 or 39 wherein said altered part is a sepal, bract, petiole, peduncle, ovary or anther stem.

41. The genetically modified plant or part thereof or cells therefrom of claim 36 or 37 or 38 or 39 wherein said altered part is a leaf, root, flower, seed, fruit, nut, berry or vegetable.

42. The genetically modified plant or part thereof or cells therefrom of claim 36 wherein the plant is selected from a rose, carnation, lisianthus, petunia, lily, pansy, gerbera, chrysanthemum, *Torenia*, *Begonia*, *Cyclamen*, *Nierembergia*, *Catharanthus*, *Pelargonium*, *Orchid*, grape, *Euphorbia* or *Fuchsia*.

43. Flowers cut or severed from a plant of claim 27.

44. Progeny, offspring of progeny or vegetation propagates lines of the genetically modified plant of any one of claims 27 to 42.

45. An extract from a plant or plant part of claim 27.

46. The extract of claim 45 wherein the extract is a flavoring or food additive or health product or beverage or juice or coloring.

47. A method for producing a genetically modified plant capable of synthesizing FMT, said method comprising stably transforming a cell of a suitable plant with a nucleic acid molecule which comprises a sequence of nucleotides encoding or complementary to a sequence encoding an FMT of any one of claims 1 to 3 or 9 or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof under conditions permitting the eventual expression of said nucleic acid sequence, regenerating a transgenic plant from the cell and growing said transgenic plant for a time and under conditions sufficient to permit the expression of the nucleic acid sequence.

48. A method for producing a genetically modified plant with reduced indigenous or existing FMT activity, said method comprising stably transforming a cell of a suitable plant with a nucleic acid molecule which comprises a sequence of nucleotides encoding or complementary to a sequence encoding an FMT of any one of claims 1 to 3 or 9 or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof, regenerating a transgenic plant from the cell and where necessary growing said transgenic plant under conditions sufficient to permit the expression of the nucleic acid.

49. A method for producing a genetically modified plant with reduced indigenous or existing FMT activity, said method comprising altering an FMT encoding gene through modification of the indigenous sequences *via* homologous recombination from an appropriately altered nucleic acid comprising a sequence of nucleotides encoding or complementary to a sequence encoding a FMT of any one of claims 1 to 3 or 9 or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent or homolog analogue or derivative thereof introduced into the plant cell, and regenerating the genetically modified plant from the cell.

50. A method for producing a genetically modified plant with altered levels of FMT encoded in a nucleic acid molecule of any one of claims 1 to 3 or 9, said method comprising introducing into a cell or cells of said plant a genetic sequence selected from:

(i) an antisense sequence to *FMT* mRNA;

(ii) a sense sequence to *FMT* DNA; and/or

(iii) an RNAi-inducing sequence specific for *FMT* mRNA;

and regenerating a genetically modified plant from said cell.

51. A method for producing a transgenic plant capable of expressing a recombinant gene encoding an FMT or part thereof or which carries a nucleic acid sequence which is substantially complementary to all or a part of a mRNA molecule optionally transcribable where required to effect regulation of an FMT, said method comprising stably transforming a cell of a suitable plant with the isolated nucleic acid molecule comprising a sequence of nucleotides encoding, or complementary to a sequence encoding, an FMT of any one or claims 1 to 3 or 9 or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof, where necessary under conditions permitting the eventual expression of said isolated nucleic acid molecule, and regenerating a transgenic plant from the cell.

52. A method for producing a transgenic plant of any one of claims 47 to 51 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:1;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:1;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 1 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:2;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:2;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

53. A method for producing a transgenic plant of any one of claims 47 to 51 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:4;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:4;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 4 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:5;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:5;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

54. A method for producing a transgenic plant of any one of claims 47 to 51 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:6;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:6;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 6 or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

55. A method for producing a transgenic plant of any one of claims 47 to 51 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:26;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:26;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 26 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:7;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:7;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

56. A method for producing a transgenic plant of any one of claims 47 to 51 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:11;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:11;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 11 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:12;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:12;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

57. A method for producing a transgenic plant of any one of claims 47 to 51 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:21;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:21;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 21 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:22;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:22;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

58. A method for producing a transgenic plant of any one of claims 47 to 51 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:41;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:41;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 41 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:42;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:42;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

59. A method for producing a transgenic plant of any one of claims 47 to 51 having the nucleotide sequence comprising:

(i) a nucleotide sequence set forth in SEQ ID NO:43;

(ii) a nucleotide sequence having at least about 50 % similarity after optimal alignment to SEQ ID NO:43;

(iii) a nucleotide sequence capable of hybridizing under low stringency conditions to SEQ ID NO: 43 or its complementary form;

(iv) a nucleotide sequence capable of encoding the amino acid sequence set forth in SEQ ID NO:44;

(v) a nucleotide sequence capable of encoding an amino acid sequence having at least about 50% similarity after optimal alignment to SEQ ID NO:44;

(vi) a nucleotide sequence capable of hybridizing under low stringency conditions to the nucleotide sequence in (iv) or (v) or its complementary form;

wherein said nucleotide sequence encodes a FMT molecule or a mutant, part, fragment or portion thereof or a functional and/or structural equivalent, homolog, analogue or derivative thereof.

60. The method of any one of claims 47 to 59 wherein the plant exhibits altered inflorescence.

61. An isolated oligonucleotide of at least 5 nucleotides having substantial similarity or complementarily to a part or region of a molecules with a nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:11 or SEQ ID NO:21 or SEQ ID NO:26 or SEQ ID NO:41 or SEQ ID NO:43 or having at least about 50% similarity thereto or capable of hybridising to the sequence set forth in SEQ ID NO:1 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:11 or SEQ ID NO:21 or SEQ ID NO:26 or SEQ ID NO:41 or SEQ ID NO:43 under low stringency conditions, wherein said nucleotide sequence encodes a polypeptide having FMT activity.

62. An isolated oligonucleotide sequence set forth in SEQ ID NO:3 or SEQ ID NO:8 or SEQ ID NO:9 or SEQ ID NO:10 or SEQ ID NO:13 or SEQ ID NO:14 or SEQ ID NO:15 or SEQ ID NO:16 or SEQ ID NO:17 or SEQ ID NO:18 or SEQ ID NO:19 or SEQ ID NO:20 or SEQ ID NO:23 or SEQ ID NO:24 or SEQ ID NO:25 or SEQ ID NO:27 or SEQ ID NO:28 or SEQ ID NO:29 or SEQ ID NO:30 or SEQ ID NO:31 or SEQ ID NO:32 or SEQ ID NO:33 or SEQ ID NO:34 or SEQ ID NO:35 or SEQ ID NO:36 or SEQ ID NO:37 or SEQ ID NO:38 or SEQ ID NO:39 or SEQ ID NO:40.

63. An isolated recombinant FMT encoded in a nucleic acid molecule of any one of claims 1 to 17.

64. The recombinant FMT of claim 63 wherein the recombinant FMT is a fusion molecule comprising two or more heterologous amino acid sequences.

65. An isolated recombinant FMT nucleic acid molecule of any one of claims 1 to 17 comprising a fusion of two or more heterologous nucleotide sequences.

66. A prokaryotic organism carrying a genetic sequence encoding an FMT molecule according to any one of claims 1 to 17 extrachromasomally in plasmid form.

67. A eukaryotic organism carrying a genetic sequence encoding an FMT molecule according to any one of claims 1 to 17 extrachromasomally in plasmid form.

68. Use of a nucleic acid molecule of any one of claims 1 to 17 in the manufacture of a genetically modified plant.

69. Use of claim 68 wherein the genetically modified plant exhibits altered flowers or inflorescence.

Figure 1a

Figure 1b

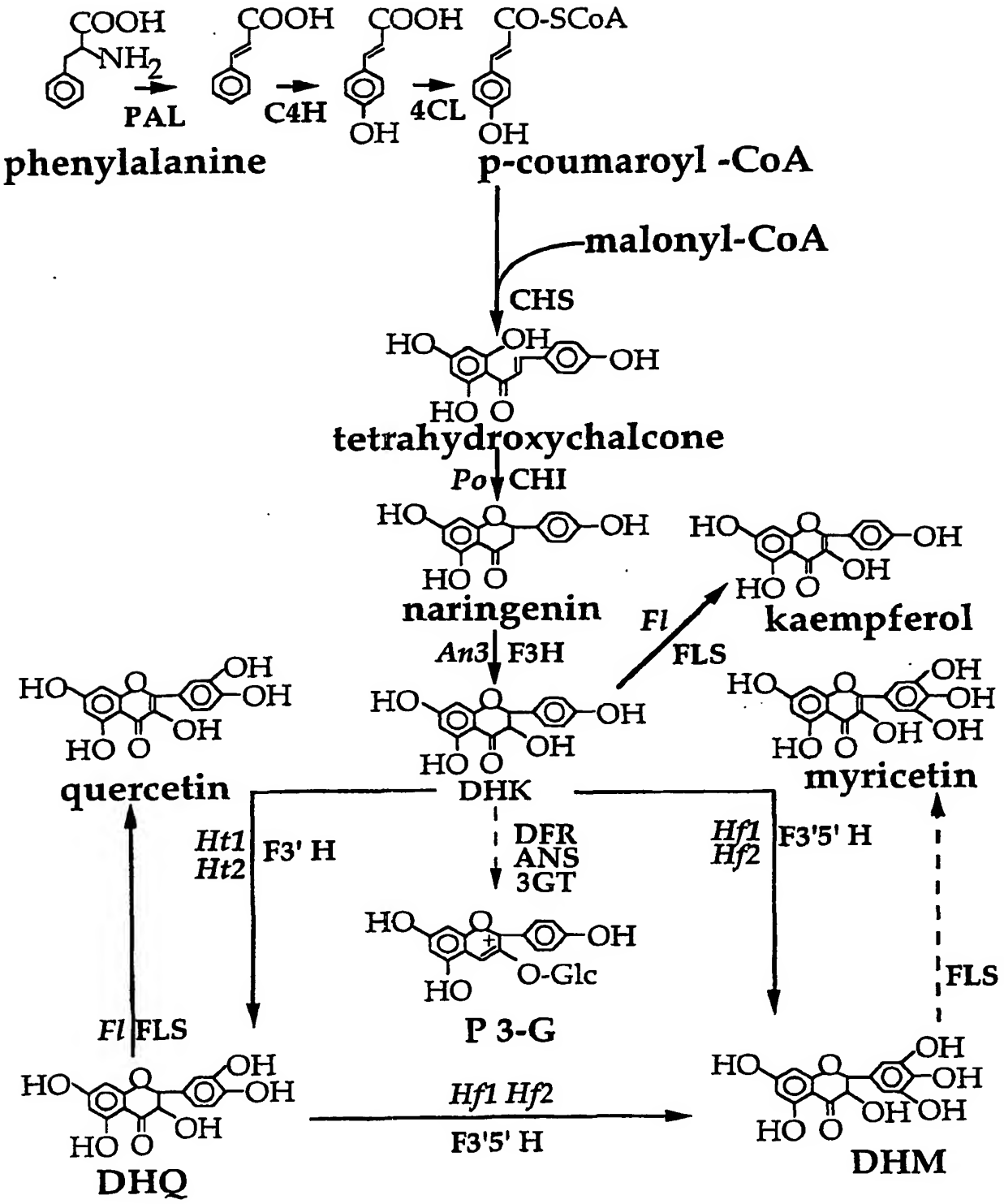

COOH
NH2
PAL
COOH
C4H
COOH
OH
4CL
CO-SCoA
OH
phenylalanine
p-coumaroyl -CoA
malonyl-CoA
CHS
tetrahydroxychalcone
Po CHI
naringenin
An3 F3H
Fl FLS
kaempferol
quercetin
DHK
myricetin
Ht1 Ht2 F3' H
DFR ANS 3GT
Hf1 Hf2 F3'5' H
O-Glc
P 3-G
Fl FLS
FLS
Hf1 Hf2
F3'5' H
DHQ
DHM

Figure 1A

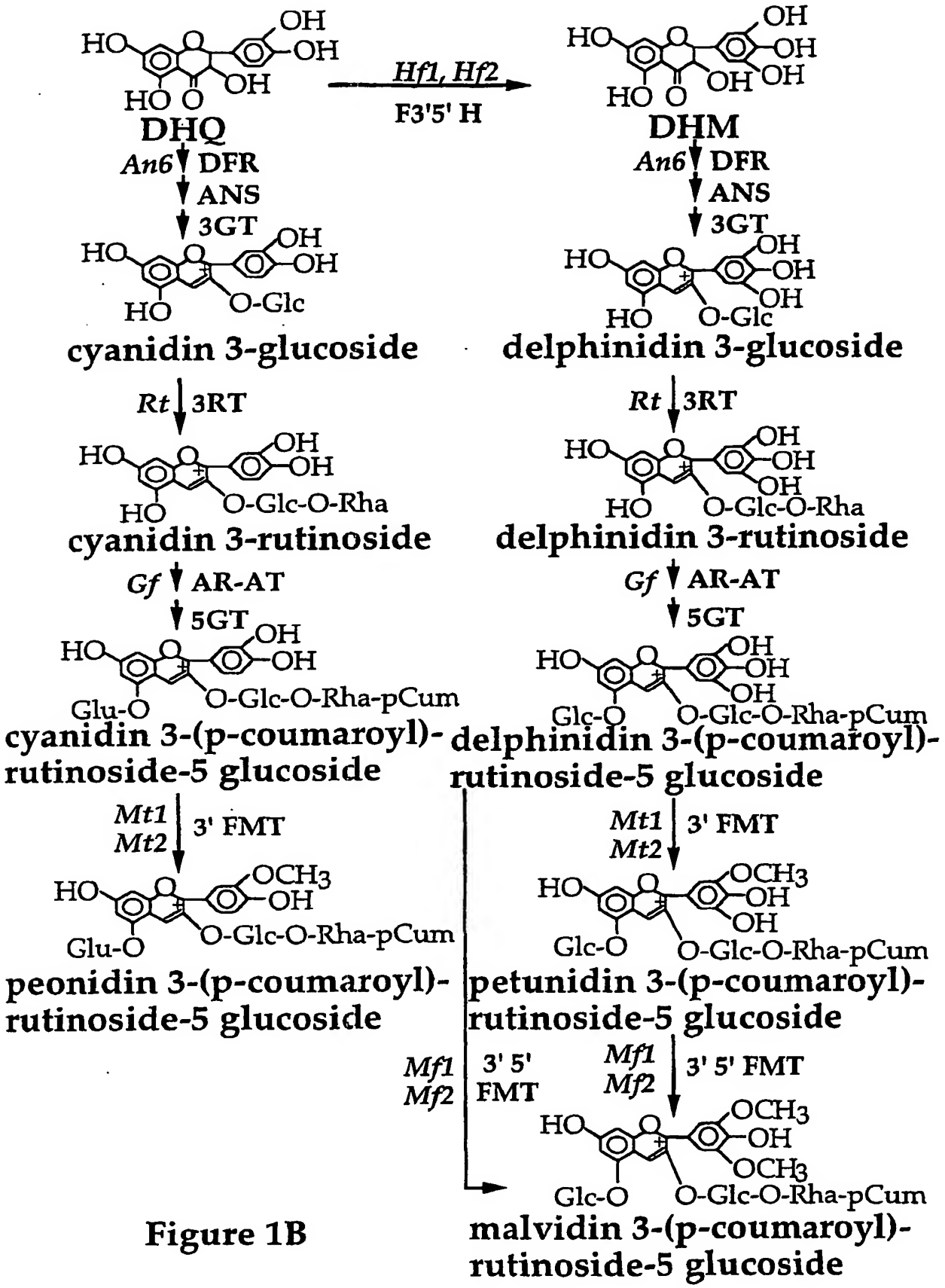
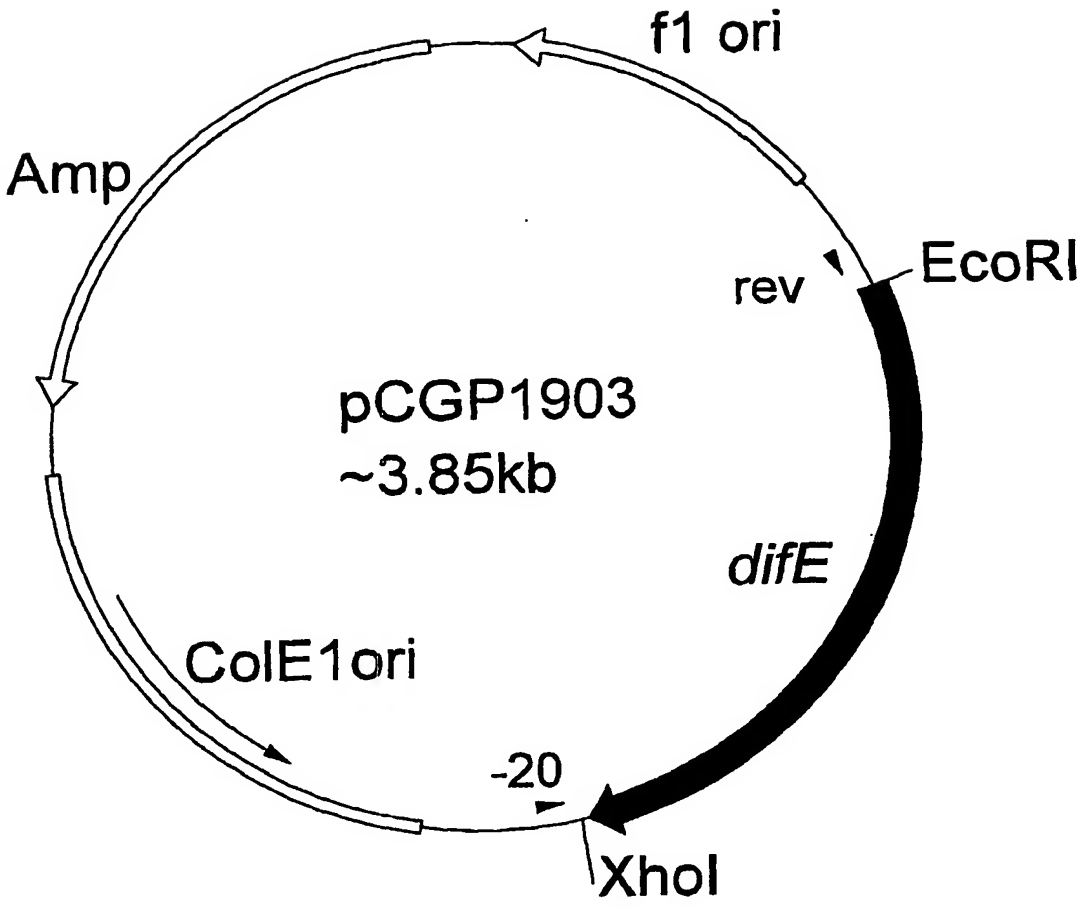

DHQ
Hf1, Hf2
F3'5' H
DHM
An6 DFR
ANS
3GT
cyanidin 3-glucoside
delphinidin 3-glucoside
Rt 3RT
cyanidin 3-rutinoside
delphinidin 3-rutinoside
Gf AR-AT
5GT
cyanidin 3-(p-coumaroyl)-rutinoside-5 glucoside
delphinidin 3-(p-coumaroyl)-rutinoside-5 glucoside
Mt1 Mt2 3' FMT
peonidin 3-(p-coumaroyl)-rutinoside-5 glucoside
petunidin 3-(p-coumaroyl)-rutinoside-5 glucoside
Mf1 Mf2 3' 5' FMT
malvidin 3-(p-coumaroyl)-rutinoside-5 glucoside

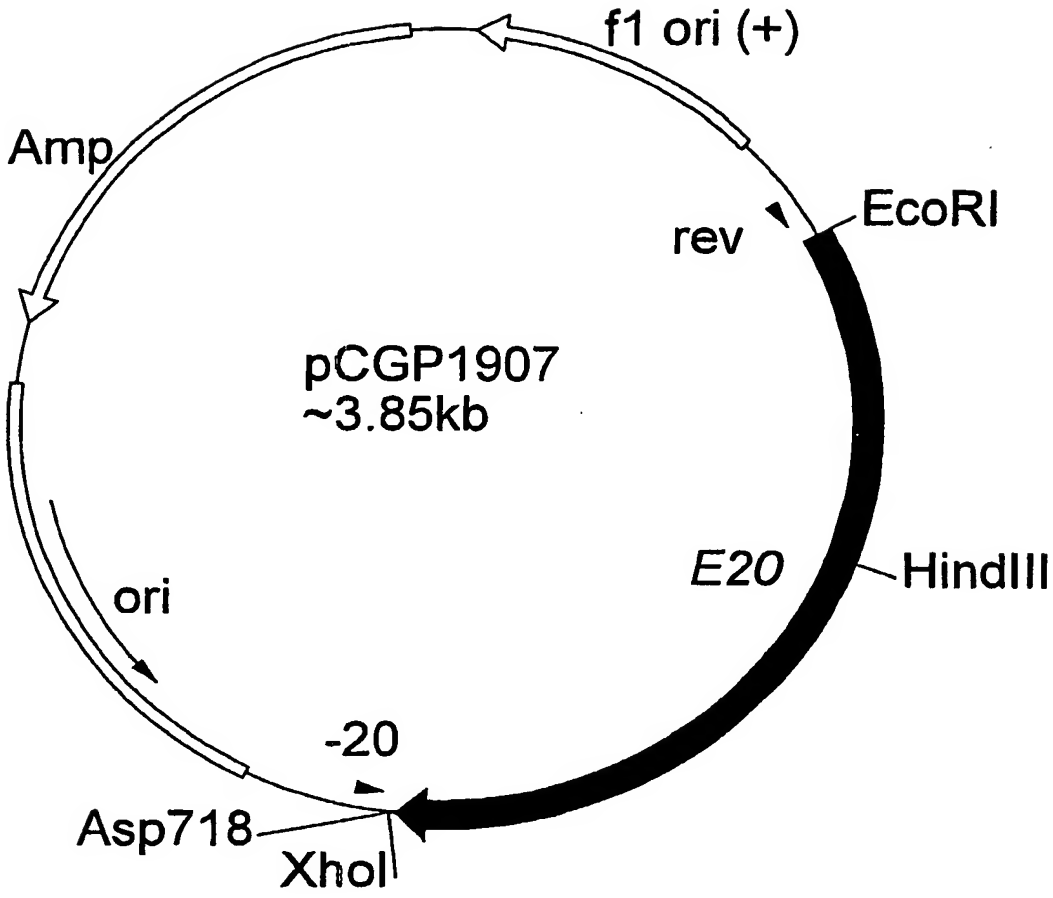
Figure 1B



Replicon: pBluescript SKII vector 2.95kb

Insert: *difE* cDNA ~0.9kb from *P. hybrida* cv. V26

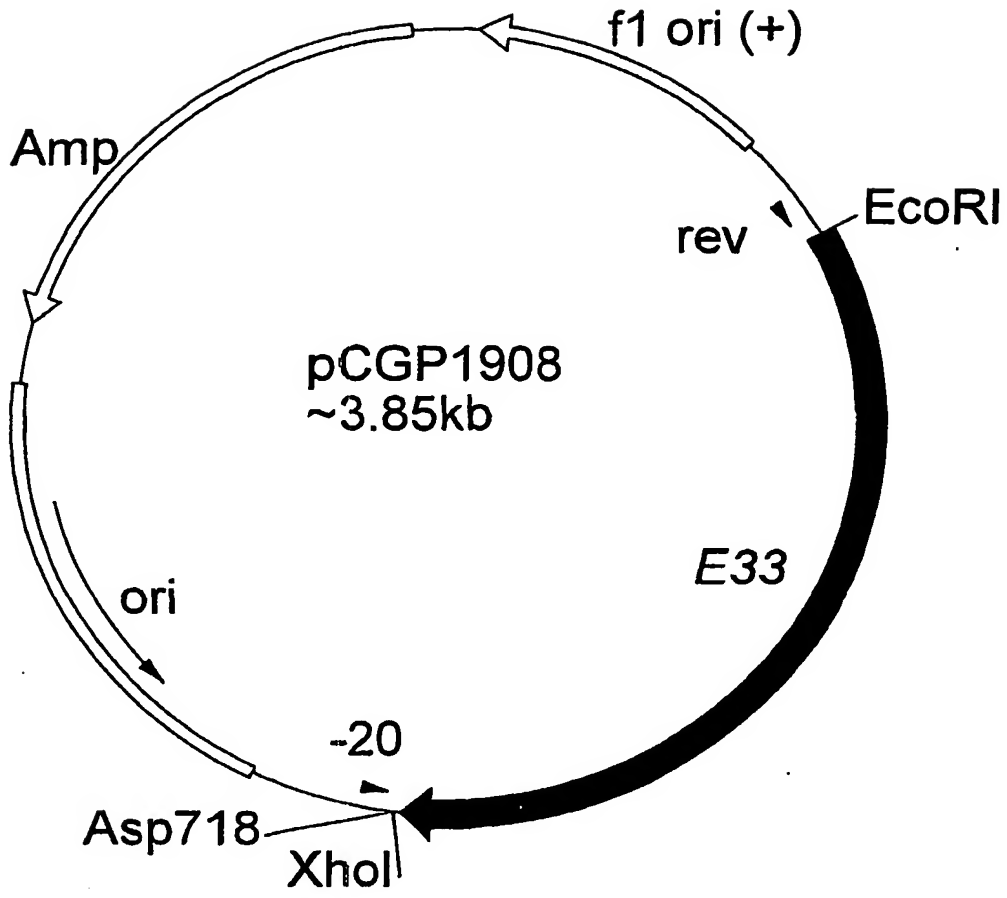
Figure 2



Replicon: pBluescript SK II (+)vector 2.95kb

Insert: *E20* cDNA ~0.9kb from *P. hybrida* cv. OGB

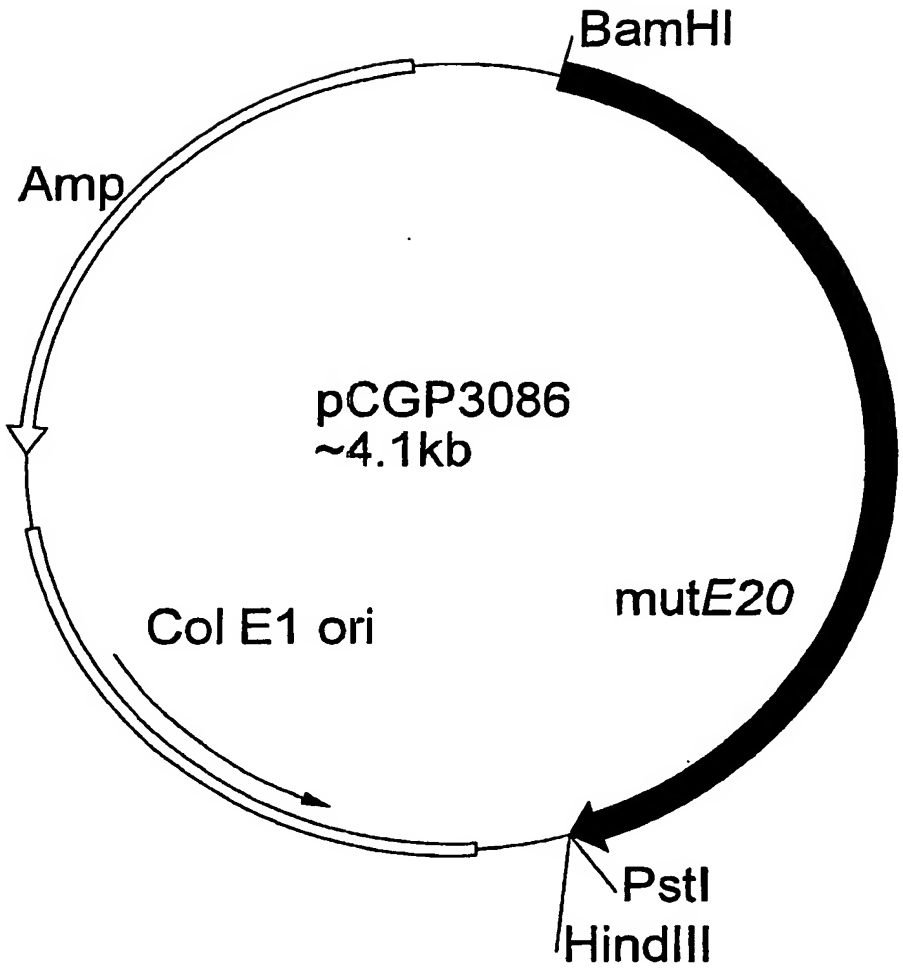
Figure 3



Replicon: pBluescript SK II (+)vector 2.95kb

Insert: *E33* cDNA ~0.9kb from
P. hybrida cv. OGB

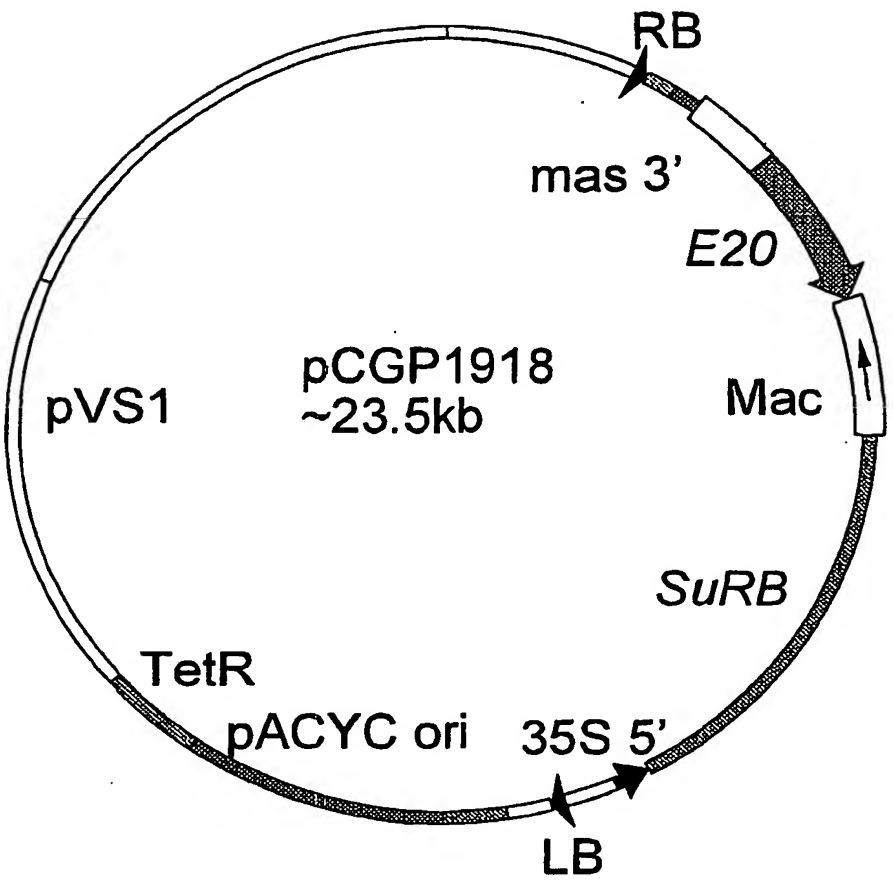
Figure 4



Replicon: pQE30 3.4kb

Insert: mut*E20* cDNA ~0.7kb from *P. hybrida* cv. OGB

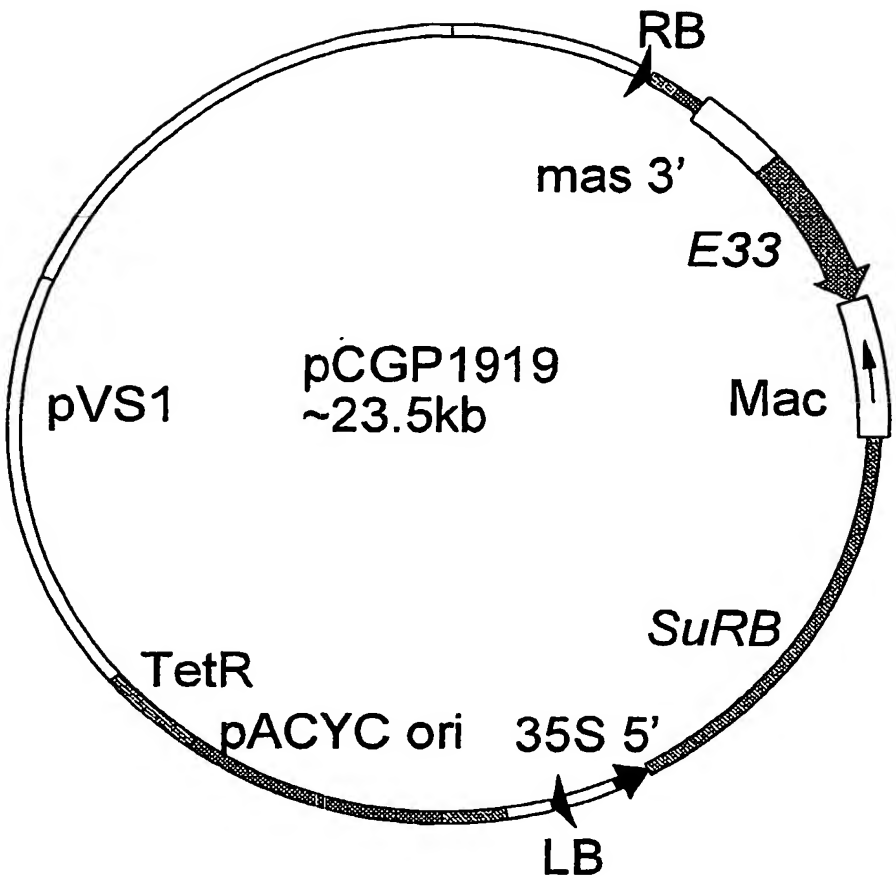
Figure 5



Replicon: pWTT2132 SmaI ~20.7kb

Insert: ~2.8kb Bgl II (blunted) fragment from pCGP1910

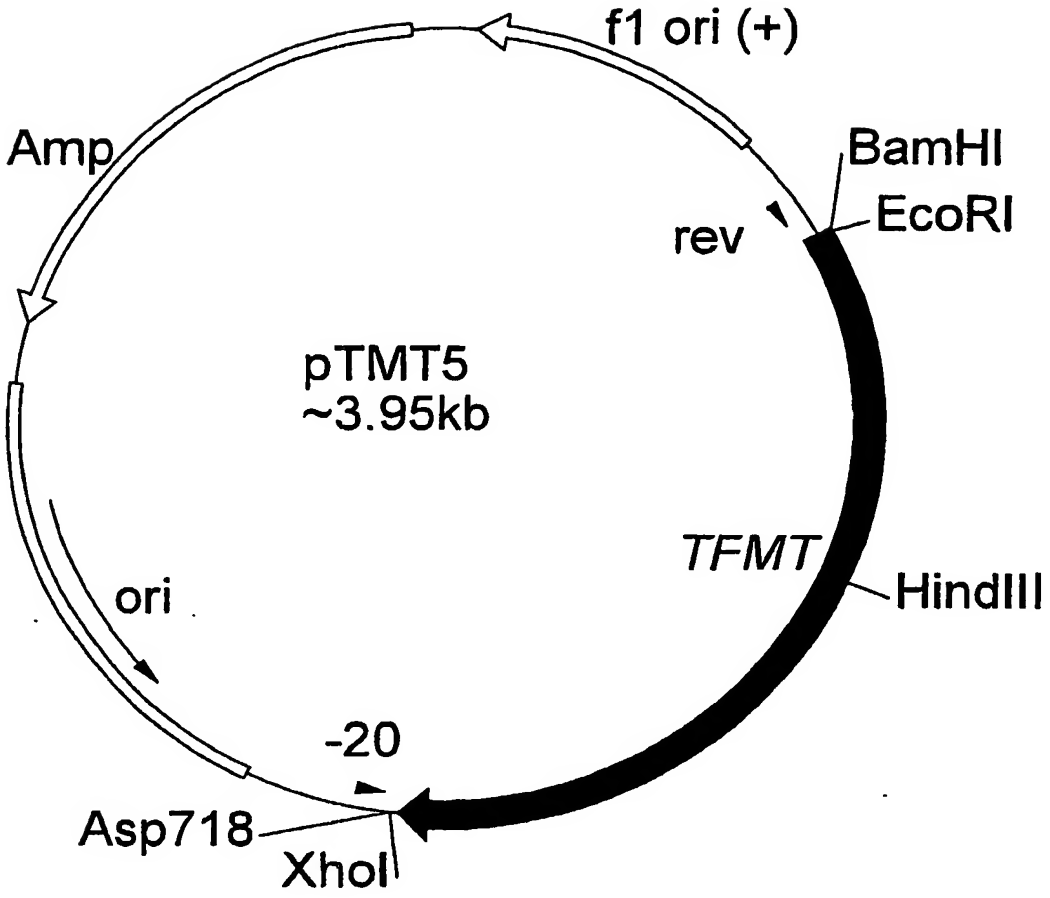
Figure 6



Replicon: pWTT2132 SmaI ~20.7kb

Insert: ~2.8kb Bgl II (blunted) fragment from pCGP1911

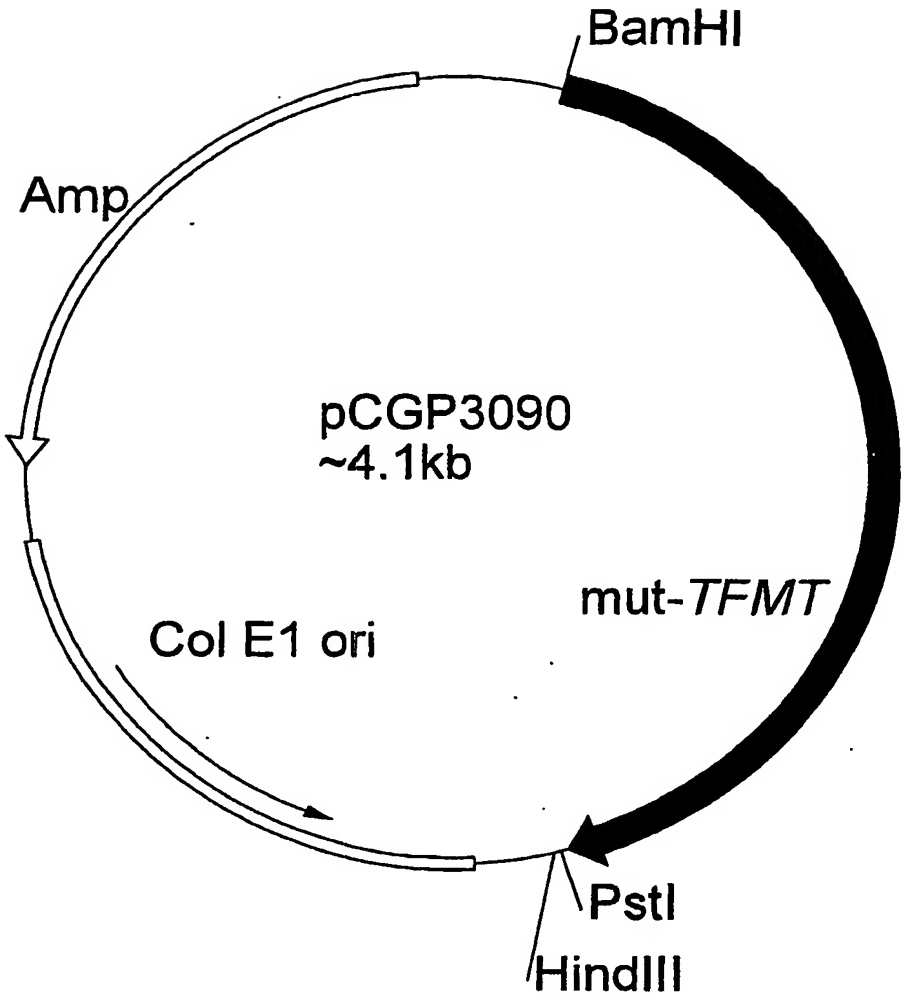
Figure 7



Replicon: pBluescript SK II (+)vector 2.95kb

Insert: *TFMT* cDNA ~1kb from
Torenia hybrida cv. Summerwave

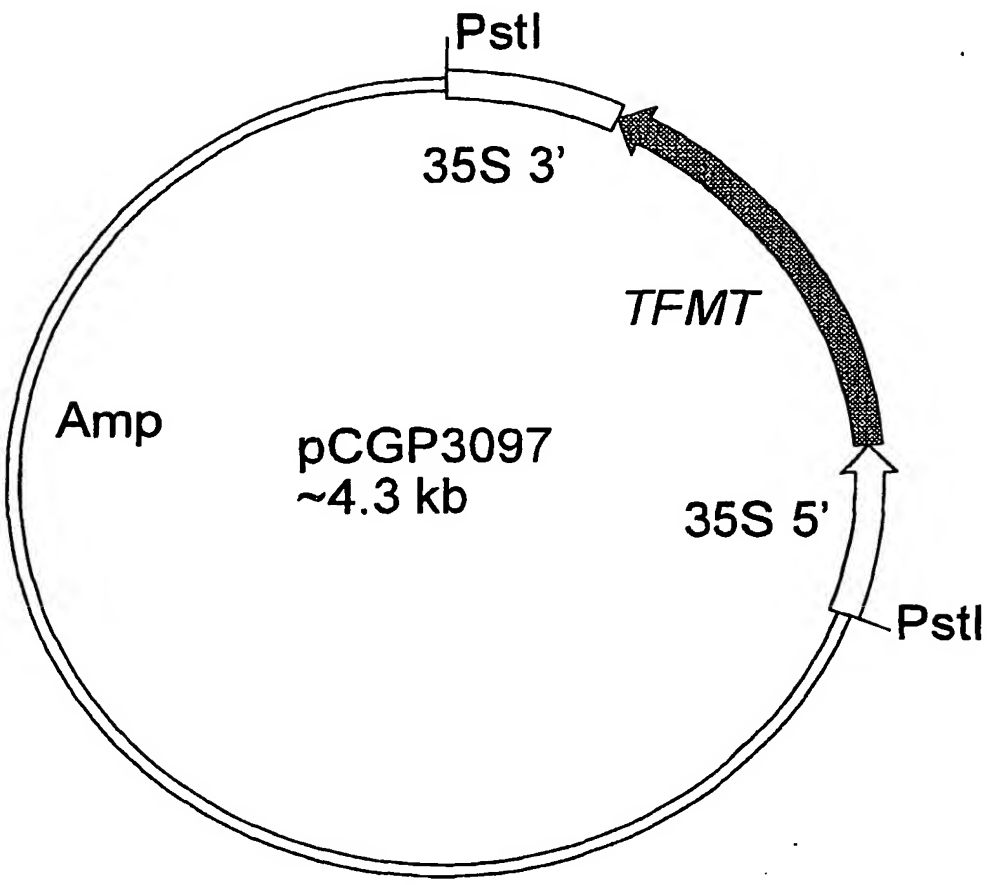
Figure 8



Replicon: pQE30 3.4kb

Insert: mut-*TFMT* cDNA ~0.7kb from *Torenia hybrida* cv. Summerwave

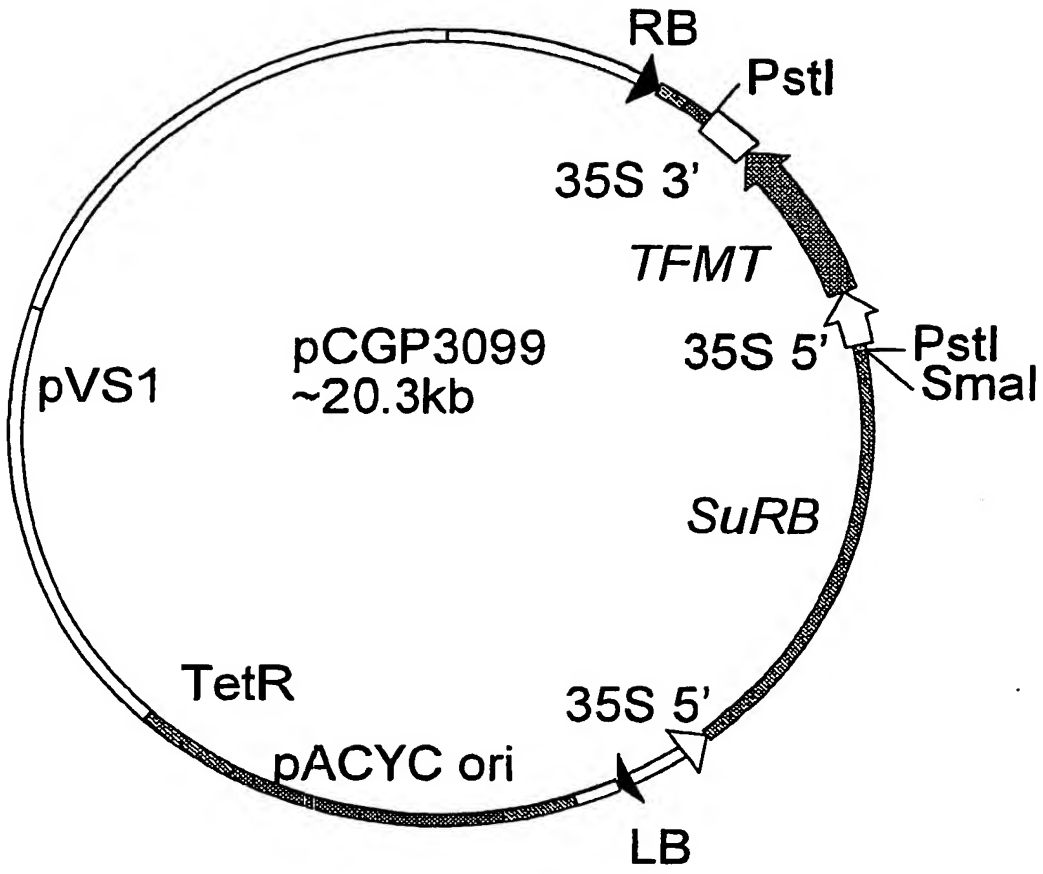
Figure 9



Replicon: pRTppoptc XbaI (blunted)/EcoRI 3.3kb

Insert: ~1.0kb Asp718 (blunted)/EcoRI fragment from pTMT5

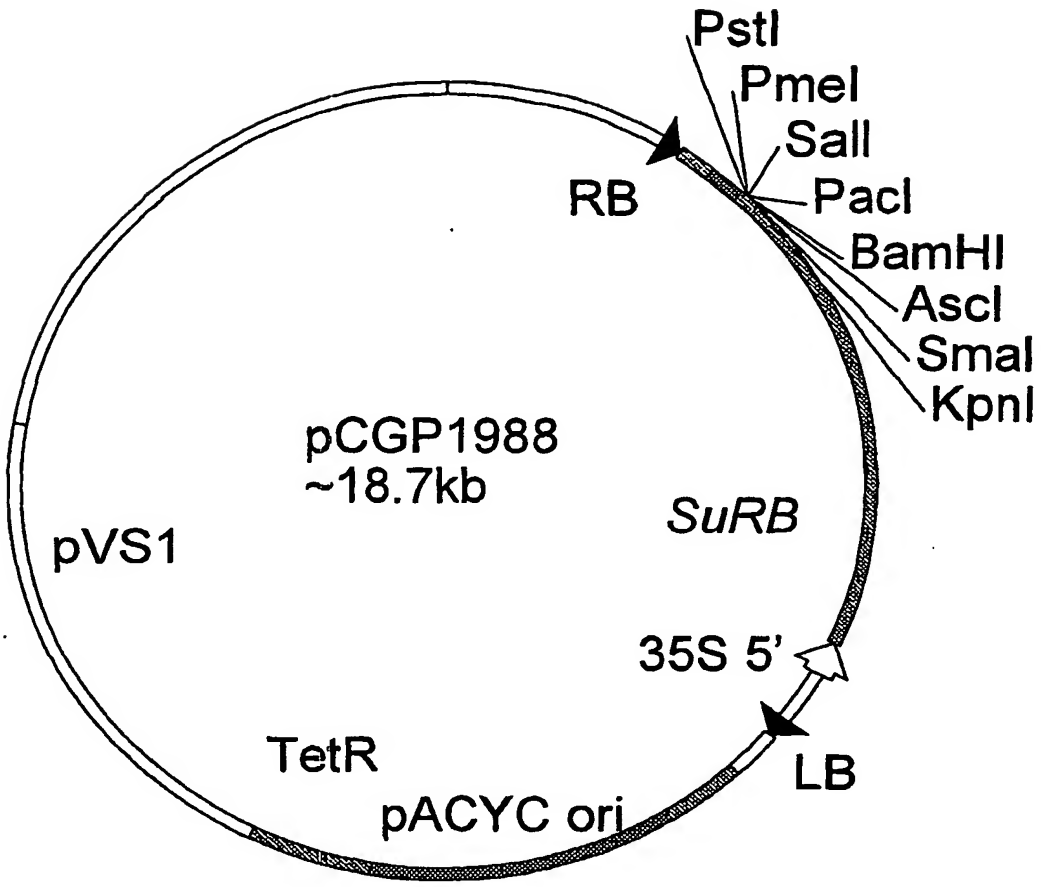
Figure 10



Replicon: pCGP1988 PstI ~18.6kb

Insert: ~1.7kb PstI fragment from pCGP3097

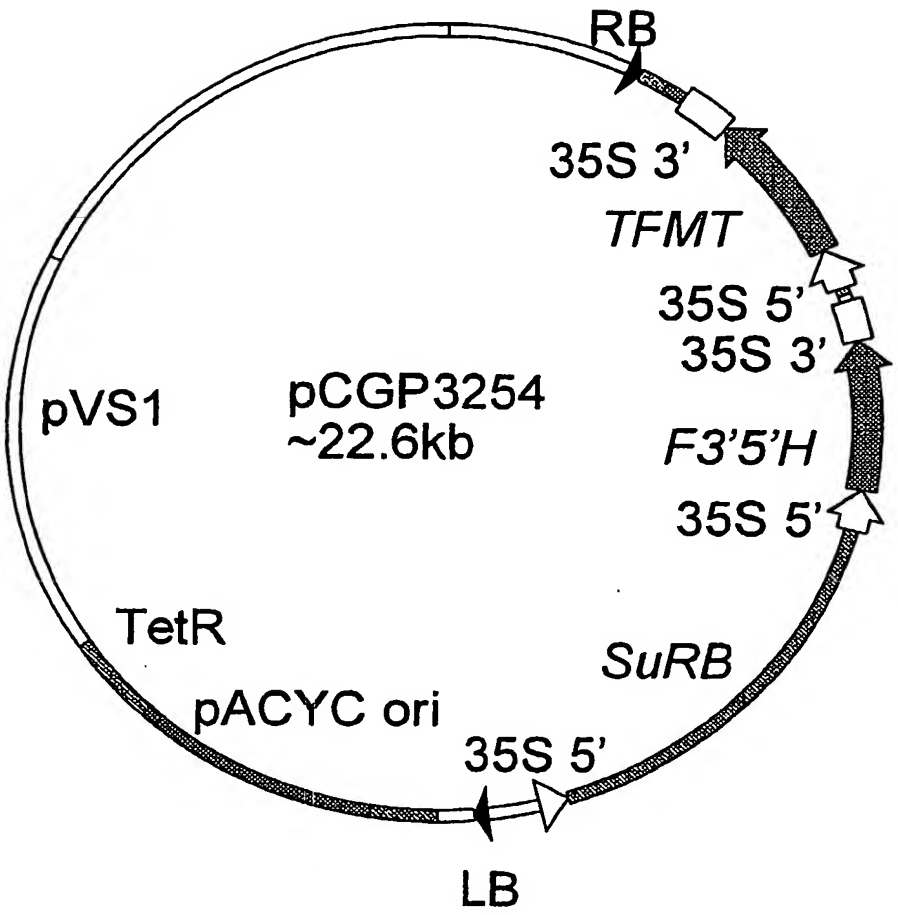
Figure 11



Replicon: pWTT2132 ~18.7kb

Insert: Multi-cloning region from pNEB193

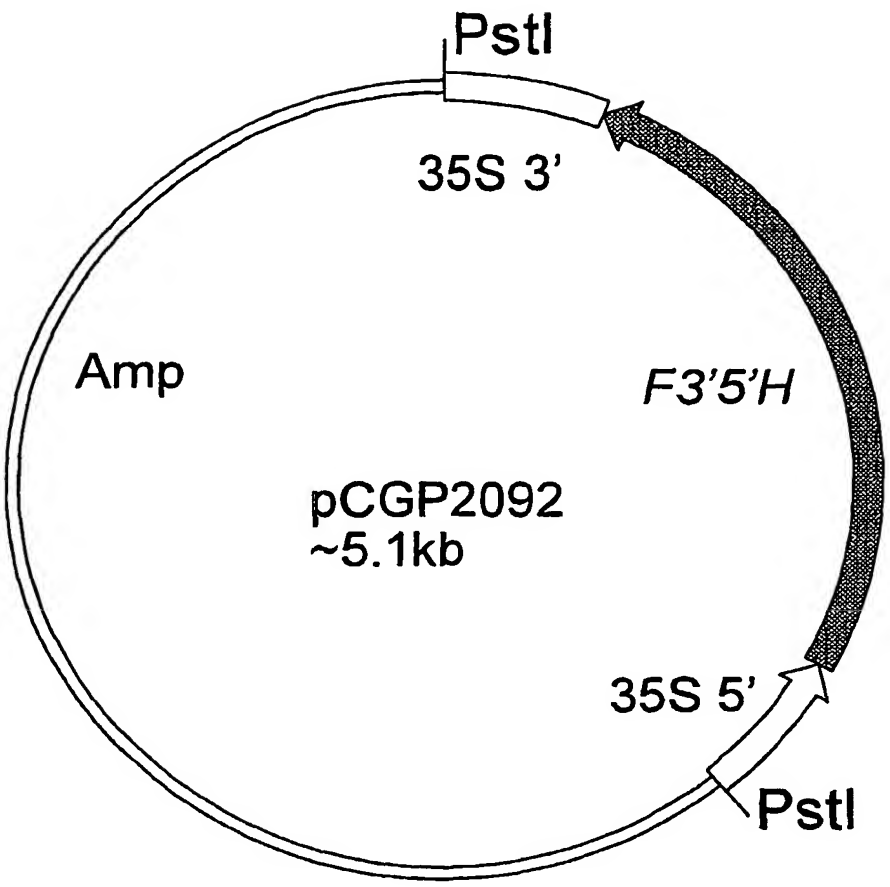
Figure 12



Replicon: pCGP3099 SmaI ~20.2kb

Insert: ~2.4kb PstI(blunted) fragment from pCGP2092

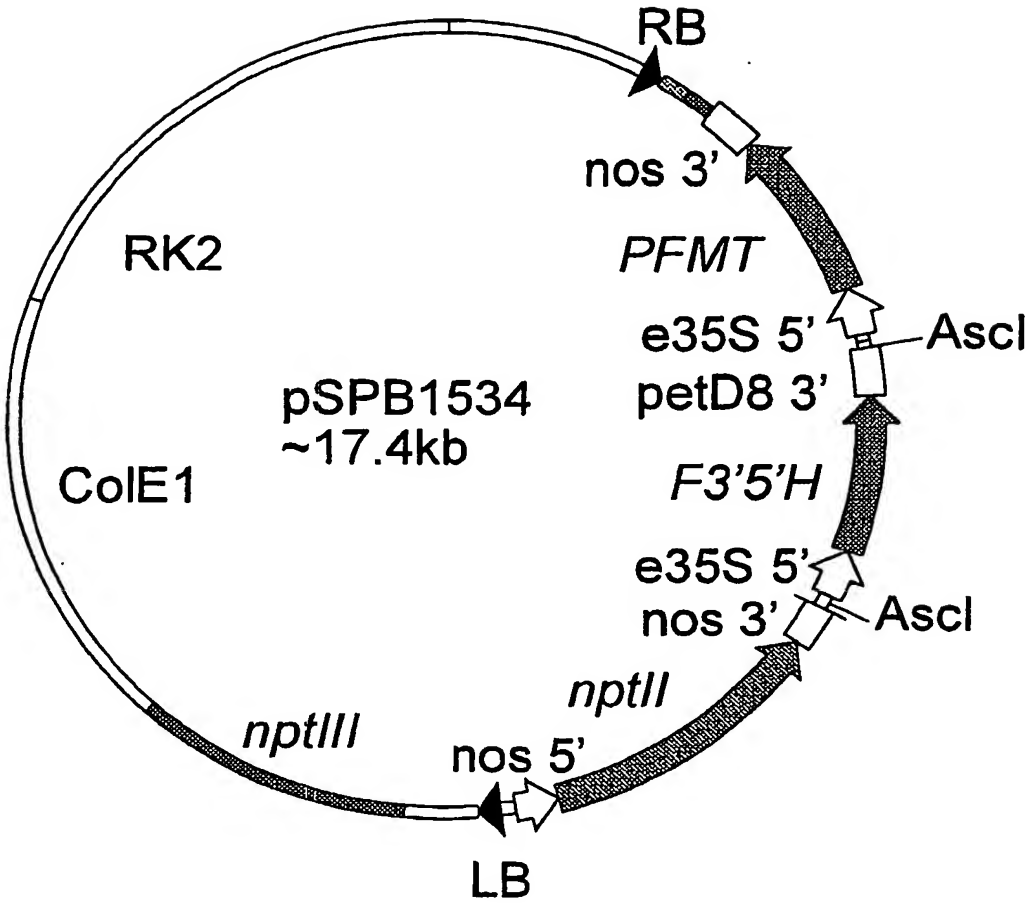
Figure 13



Replicon: pRTppoptc XbaI (blunted)/EcoRI ~3.3kb

Insert: ~1.8kb Asp718 (blunted)/EcoRI fragment from pCGP1961

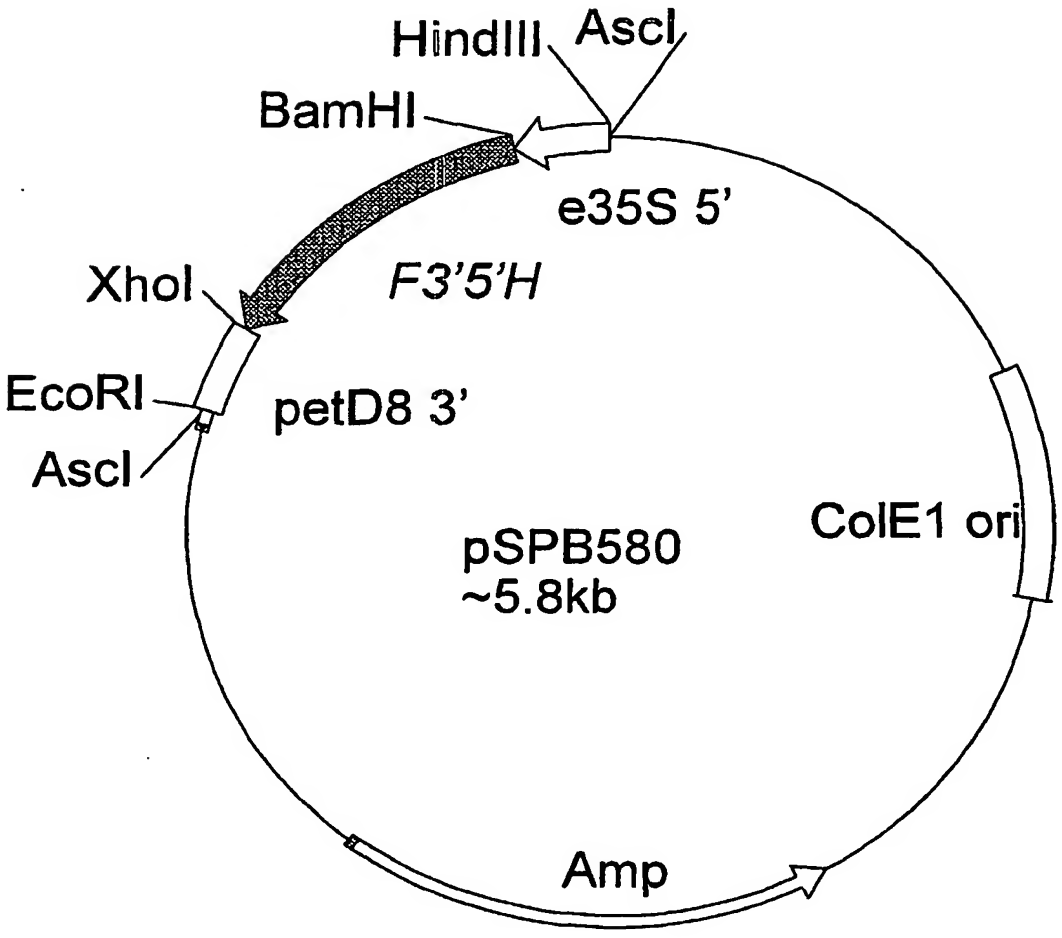
Figure 14



Replicon: pSPB1531 AscI ~14.3kb

Insert: ~3.1kb AscI fragment from pSPB580

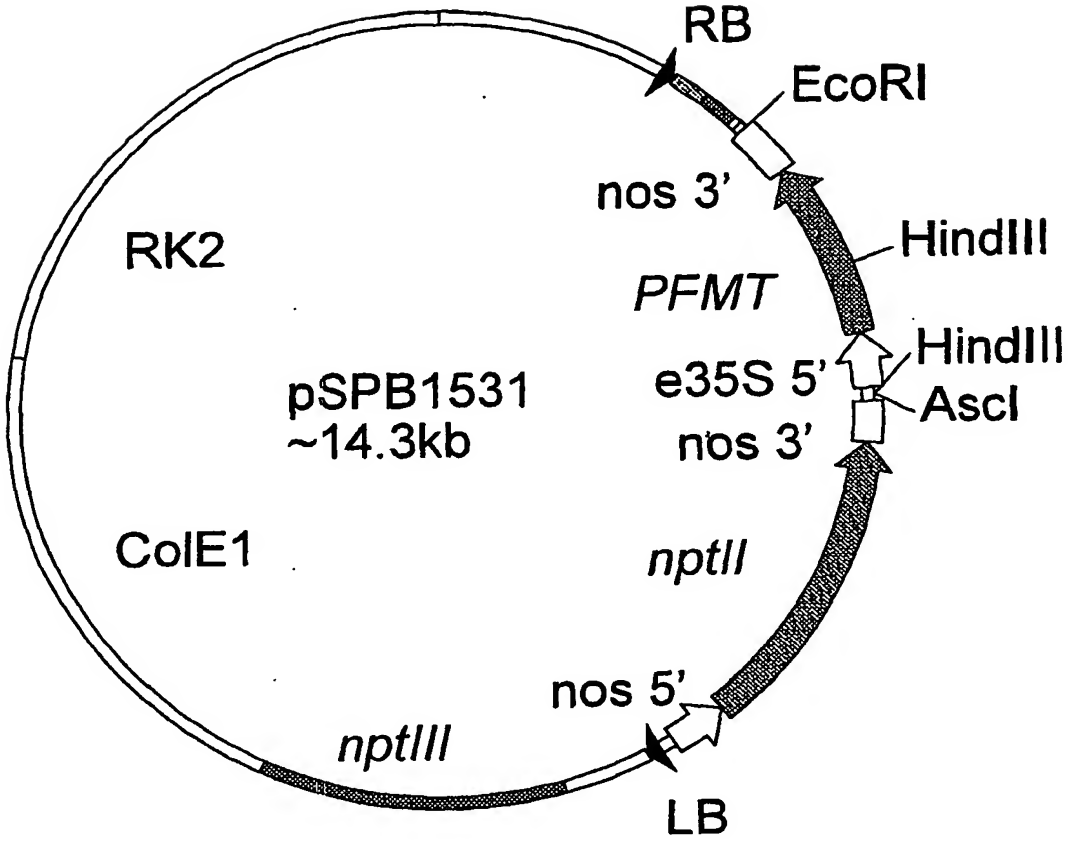
Figure 15



Replicon: pUCAPAsc BamHI/EcoRI ~2.7kb

Insert: e35S 5': Viola F3'5'H cDNA : petD8 3' ~3.1kb

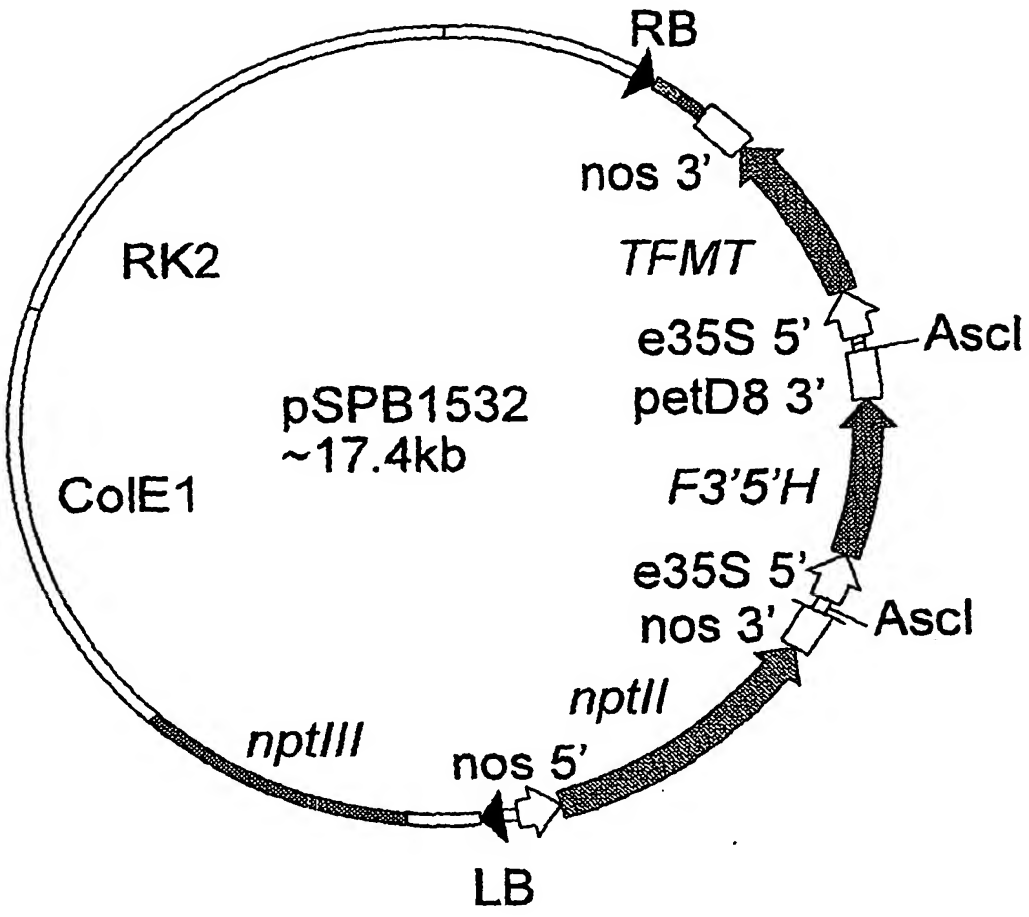
Figure 16



Replicon: pSPB176 BamHI/SalI fragment ~13.5kb

Insert: Petunia FMT cDNA BamHI/XhoI fragment (derived from pCGP1907)~0.9kb

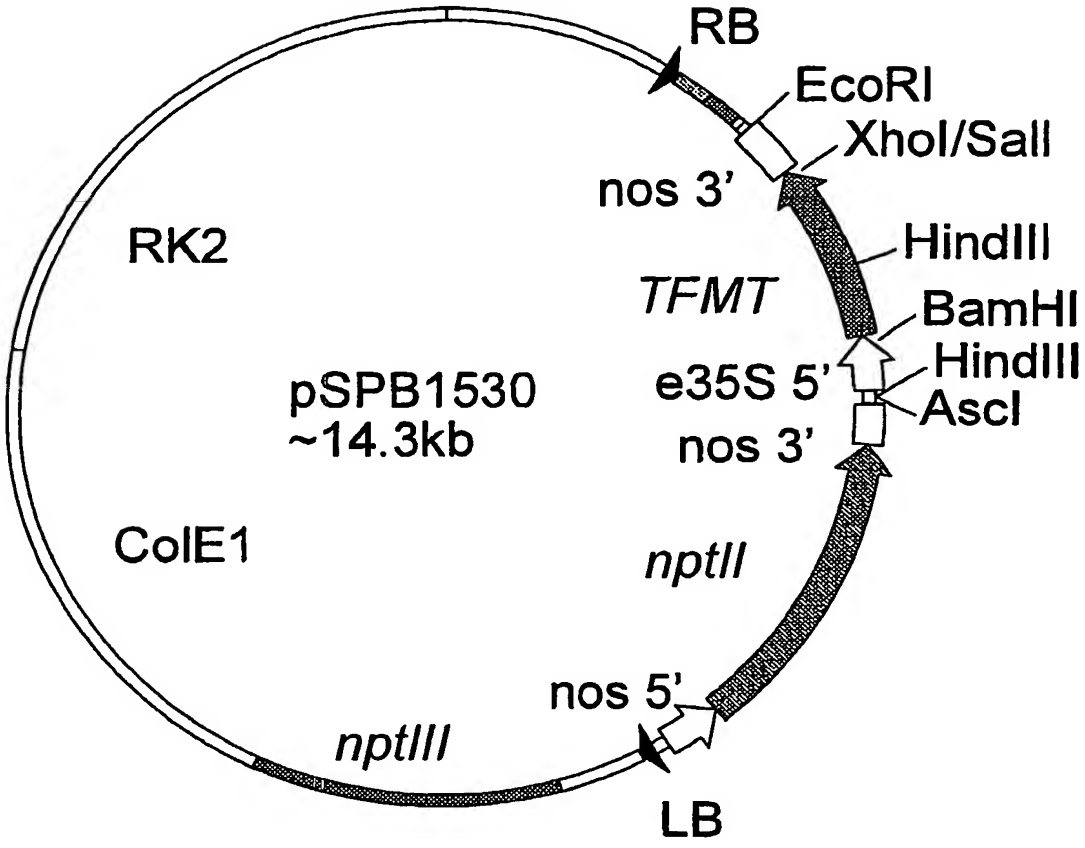
Figure 17



Replicon: pSPB1530 AscI ~14.3kb

Insert: ~3.1kb AscI fragment from pSPB580

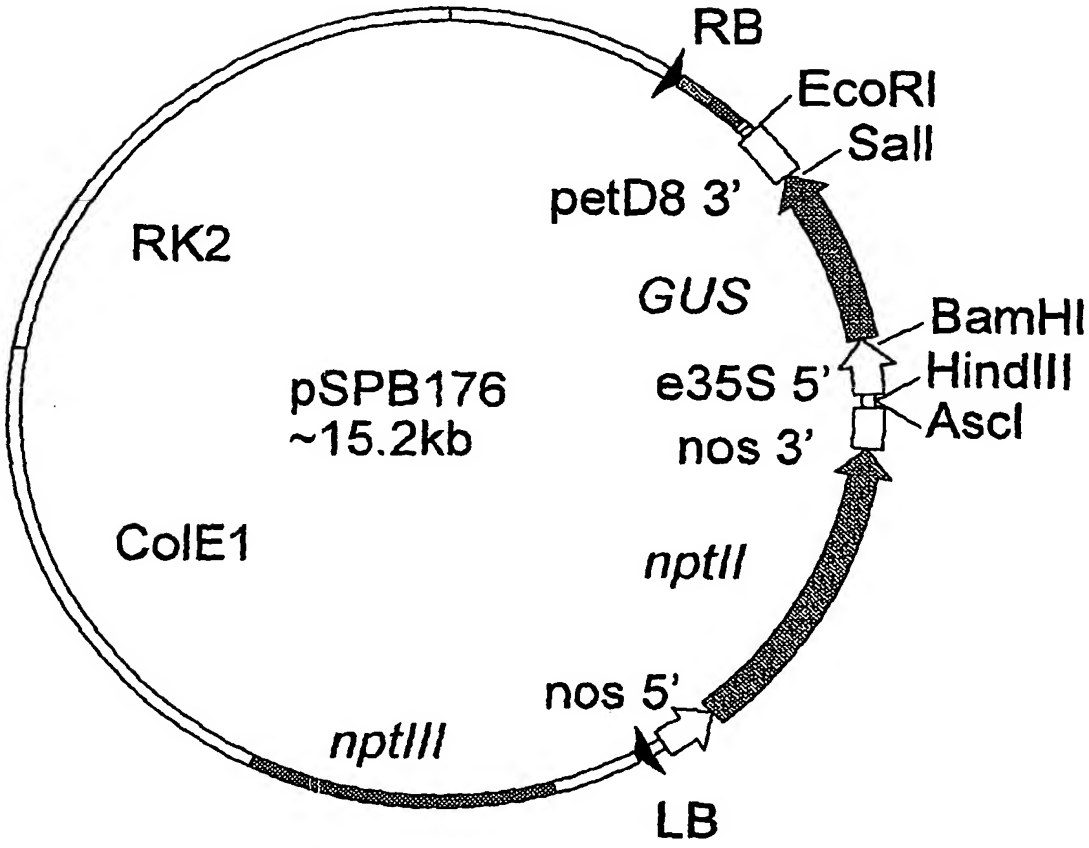
Figure 18



Replicon: pSPB176 BamHI/SalI fragment ~13.4kb

Insert: Torenia FMT cDNA BamHI/XhoI fragment (derived from pTMT5)~0.9kb

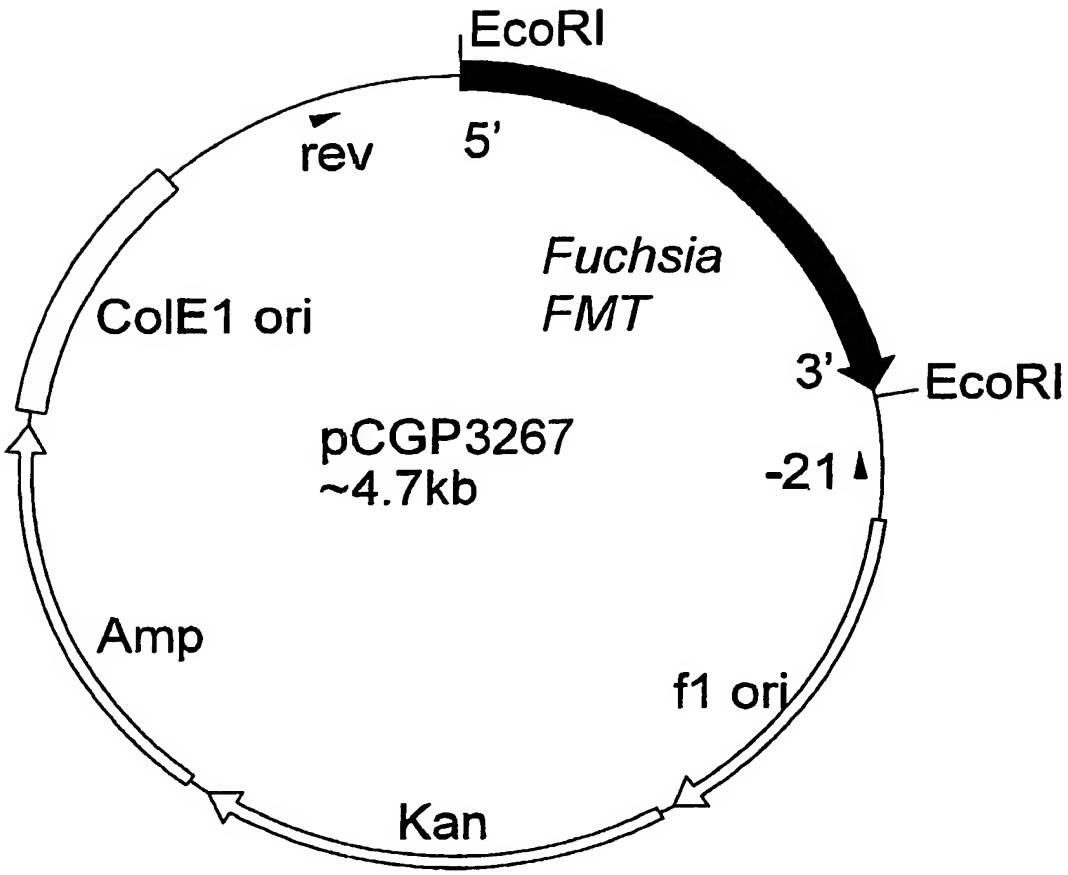
Figure 19



Replicon: pBINPLUS HindIII/EcoRI ~12.4kb

Insert: HindIII/EcoRI fragment (derived from pBE2113-ΔGUS)~2.8kb

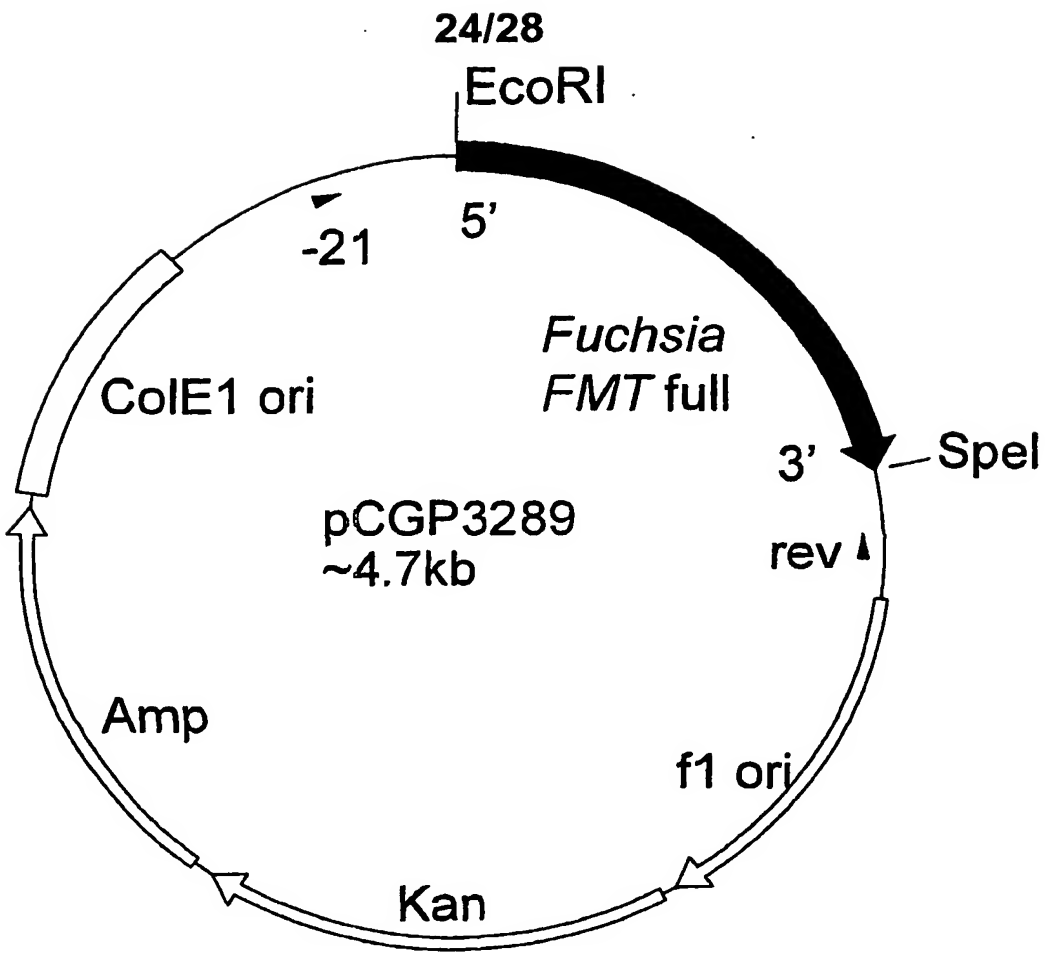
Figure 20



Replicon: pCR 2.1 3.9kb

Insert: *Fuchsia FMT* 3'RACE ~0.8kb fragment

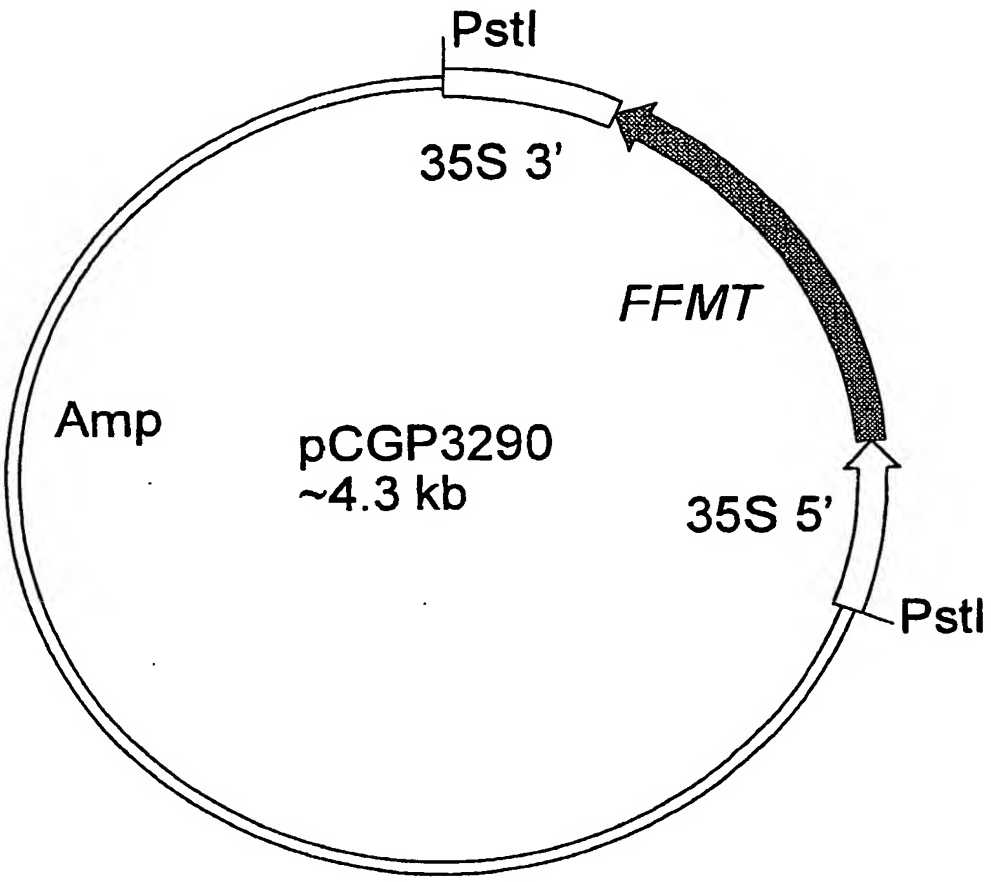
Figure 21



Replicon: pCR 2.1 3.9kb

Insert: *Fuchsia FMT* full cDNA ~1.0kb

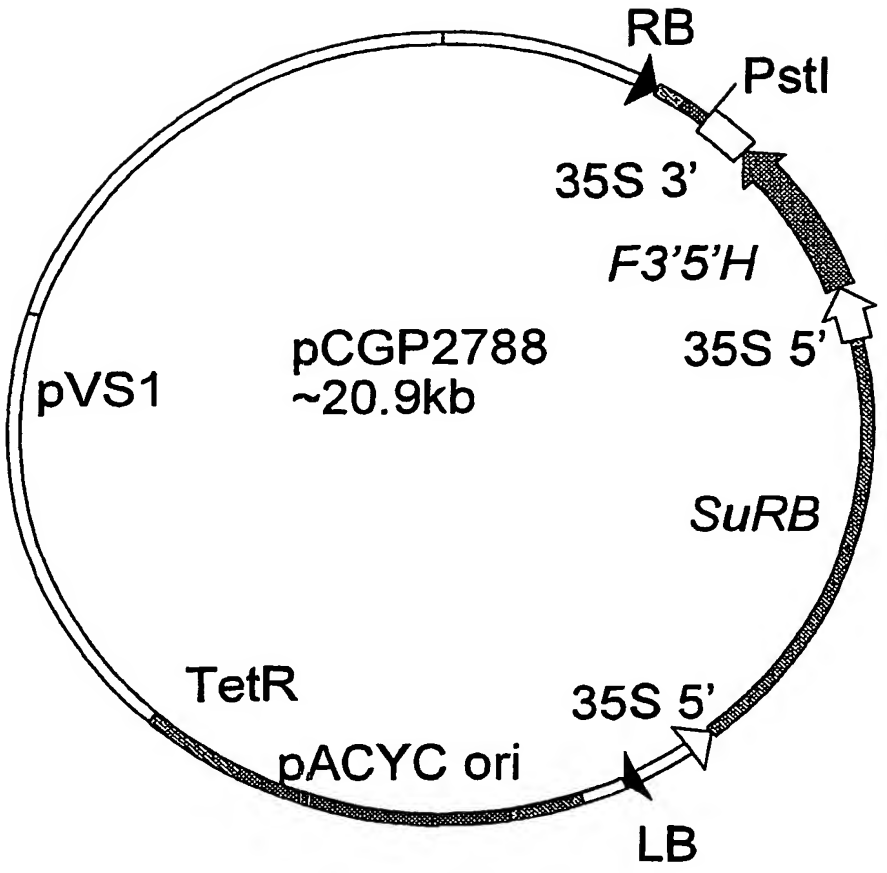
Figure 22



Replicon: pRTppoptc XbaI (blunted)/EcoRI 3.3kb

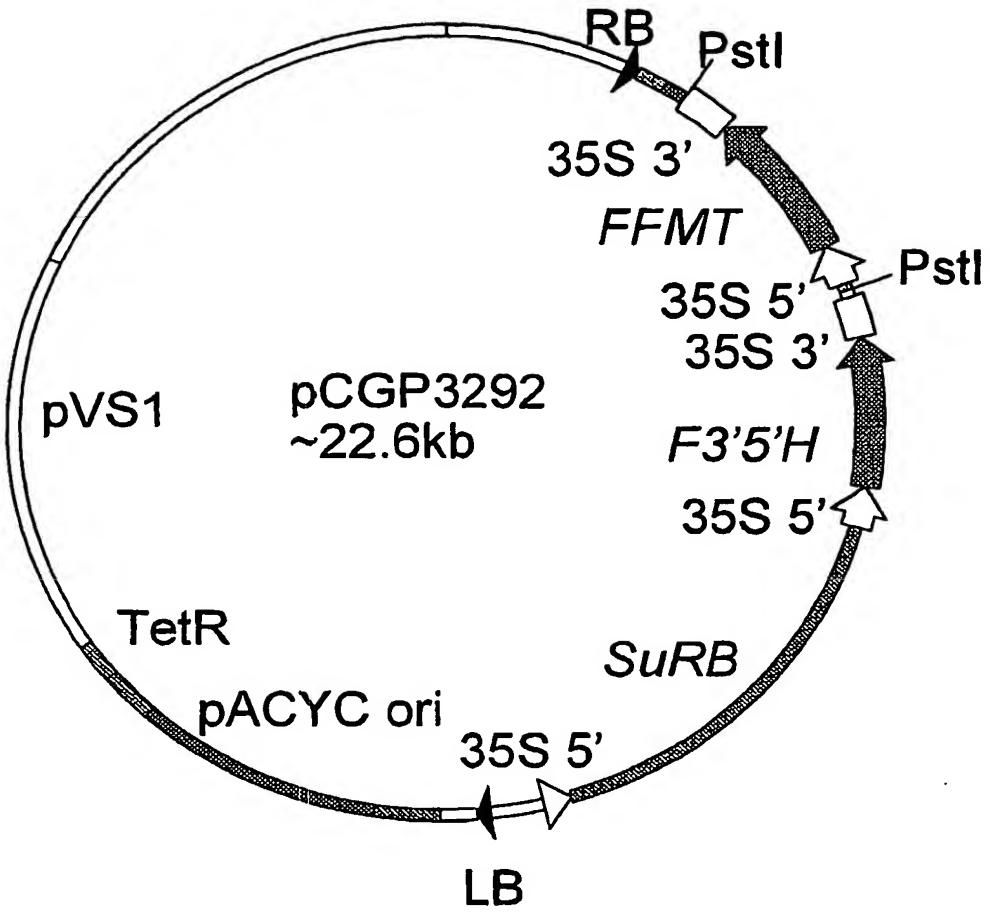
Insert: ~1.0kb SpeI (blunted)/EcoRI fragment from pCGP3289

Figure 23



Replicon: pCGP3254 PstI ~20.9kb

Figure 24



Replicon: pCGP2788 PstI ~20.9kb

Insert: ~1.7kb PstI fragment from pCGP3290

Figure 25

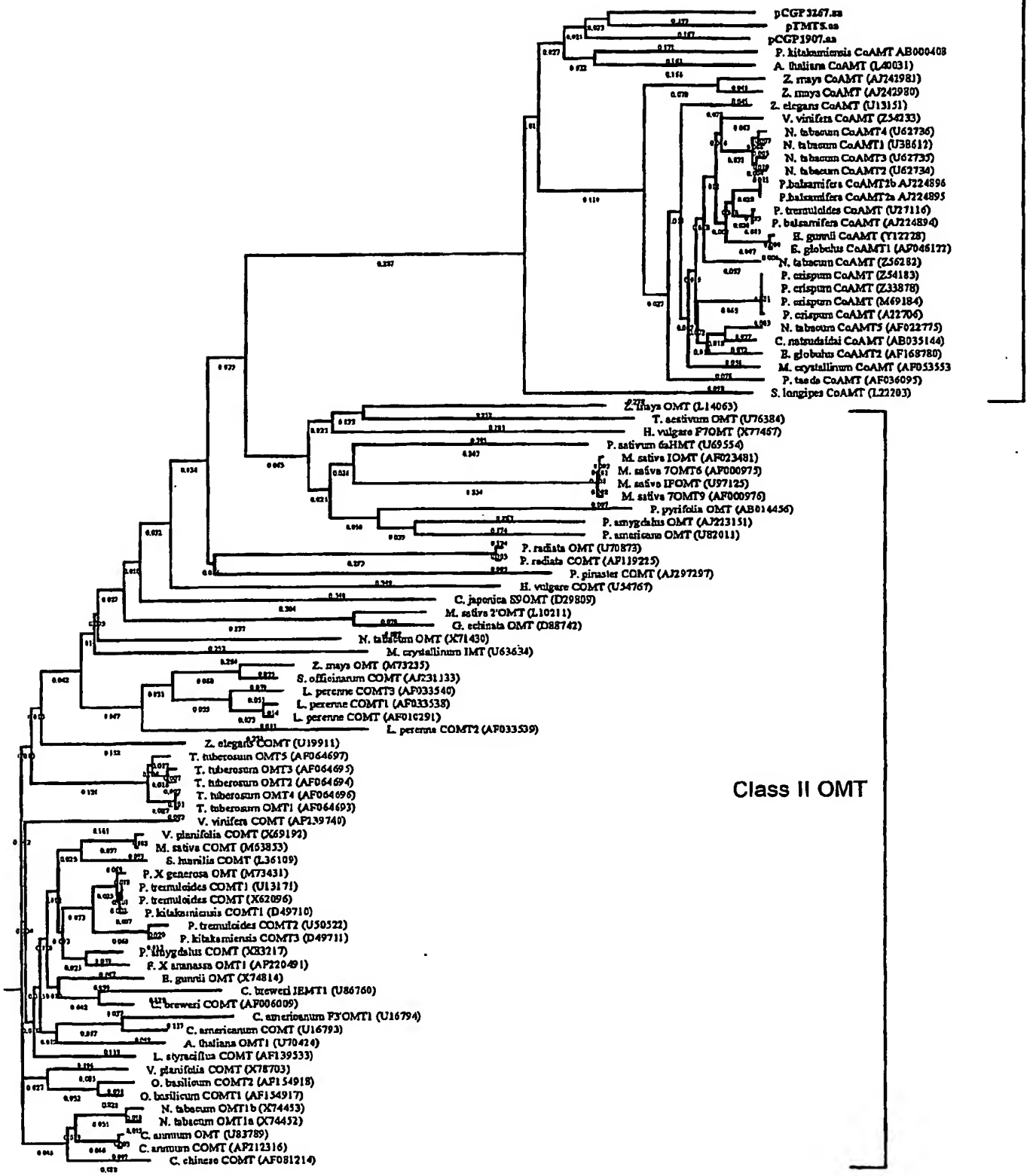

Class I OMT
pCGP3267.aa
pTMT5.aa
pCGP1907.aa
P. kitakamiensis CoAMT AB000408
A. thaliana CoAMT (L40031)
Z. mays CoAMT (AJ242981)
Z. mays CoAMT (AJ242980)
Z. elegans CoAMT (U13151)
V. vinifera CoAMT (Z54233)
N. tabacum CoAMT4 (U62736)
N. tabacum CoAMT1 (U38611)
N. tabacum CoAMT3 (U62735)
N. tabacum CoAMT2 (U62734)
P.balsamifera CoAMT2b AJ224896
P.balsamifera CoAMT2a AJ224895
P. tremuloides CoAMT (U27116)
P. balsamifera CoAMT (AJ224894)
E. gunnii CoAMT (Y12728)
E. globchus CoAMT1 (AF046122)
N. tabacum CoAMT (Z56282)
P. crispum CoAMT (Z54183)
P. crispum CoAMT (Z33878)
P. crispum CoAMT (M69184)
P. crispum CoAMT (A22706)
N. tabacum CoAMT5 (AF022775)
C. matsudaidai CoAMT (AB035144)
E. globulus CoAMT2 (AF168780)
M. crystallinum CoAMT (AF053553)
P. taeda CoAMT (AF036095)
S. longipes CoAMT (L22203)
Z. mays OMT (L14063)
T. aestivum OMT (U76384)
H. vulgare F7OMT (X77457)
P. sativum 6aHMT (U69554)
M. sativa IOMT (AF023481)
M. sativa 7OMT6 (AF000975)
M. sativa IFOMT (U97125)
M. sativa 7OMT9 (AF000976)
P. pyrifolia OMT (AB014456)
P. amygdalus OMT (AJ223151)
P. americana OMT (U82011)
P. radiata OMT (U70873)
P. radiata COMT (AF119225)
P. pinaster COMT (AJ297297)
H. vulgare COMT (U54767)
C. japonica 8'OMT (D29809)
M. sativa 2'OMT (L10211)
G. echinata OMT (D88742)
N. tabacum OMT (X71430)
M. crystallinum IMT (U63634)
Z. mays OMT (M73235)
S. officinarum COMT (AJ231133)
L. perenne COMT3 (AF033540)
L. perenne COMT1 (AF033538)
L. perenne COMT (AF016291)
L. perenne COMT2 (AF033539)
Z. elegans COMT (U19911)
T. tuberosum OMT5 (AF064697)
T. tuberosum OMT3 (AF064695)
T. tuberosum OMT2 (AF064694)
T. tuberosum OMT4 (AF064696)
T. tuberosum OMT1 (AF064693)
V. vinifera COMT (AF239740)
V. planifolia COMT (X69192)
M. sativa COMT (M53853)
S. humilis COMT (L36109)
P. X generosa OMT (M73431)
P. tremuloides COMT1 (U13171)
P. tremuloides COMT (X62096)
P. kitakamiensis COMT1 (D49710)
P. tremuloides COMT2 (U50522)
P. kitakamiensis COMT3 (D49711)
P. amygdalus COMT (X83217)
P. X euramassa OMT1 (AF220491)
E. gunnii OMT (X74814)
C. breweri IEMT1 (U86760)
C. breweri COMT (AF006009)
C. americanum F5'OMT1 (U16794)
C. americanum COMT (U16793)
A. thaliana OMT1 (U70424)
L. styraciflua COMT (AF139533)
V. planifolia COMT (X78703)
O. basilicum COMT2 (AF154918)
O. basilicum COMT1 (AF154917)
N. tabacum OMT1b (X74453)
N. tabacum OMT1a (X74452)
C. annuum OMT (U83789)
C. annuum COMT (AF212316)
C. chinese COMT (AF081214)
Class II OMT

Figure 26

SEQUENCE LISTING

<110> International Flower Developments Pty Ltd
Brugliera, Filippa (US only)
Demelis, Linda (US only)
Koes, Ronald (US only)
Tanaka, Yoshikazu (US only)

<120> Genetic sequences and uses therefor

<130> 2606090/EJH

<140> Not yet available
<141> 2003-01-24

<150> AU PS0174/02
<151> 2002-01-25

<160> 47

<170> PatentIn version 3.1

<210> 1
<211> 969
<212> DNA
<213> DifE

<400> 1
ctcaatttga atattactac caaggatttt actgaagcaa tgacaggcaa aaccgcccac 60

cctggcattc ttcgaagtga tgccctcagg aagtacattt tggaaacctc agtttatcca 120

agagagcatg agctactcaa agaactaaca aaagcttcat tcgagaatta taaagcagcg 180

agctttatgg gtcttcctca agatgaagcc cagtttctat cgatgttcct aaagctcata 240

aatgcaaaga aaacactaga gattggagtt ttcactggtt actctctgct tgttactgct 300

cttgctttgc cagaagatgg gaaagtaata gcaattgacc cggacagaga ggcatatgag 360

gttggattac cttatattca gaaggctggt gtggaacata agatcgagtt cattcaatca 420

gaagccgtgc ccgttcttga aaaactcctc tctaacgaga aagaagcagg gacatttgat 480

ttcgtgttca ttgatgctga taaggagaat tatttgaagt accatgagat agtgctaaaa 540

ttggtgaaag ttggaggagt gataggatat gacaacacct tatggtttgg gacagtggca 600

ctttcagagg atgatccaat gccagaaggt ttaagagcat taaggggaca tgttatgaag 660

gtcaatagct ttttagctac tgaccctcgt gttgaagtag ctcaactttc aattggtgat 720

ggccttaccc tttgcaggcg tctctcctag gtccagttaa ttggtgcaat gccaagtcaa 780

cgcgaagata tgtactagat gtatgtcagg ggttgaattt attgaattta tgttgttgag 840

aagaacaaaa gttctatatt tgtgttgttt gcaagtattt gaaacttgta ggagcctttt 900

ggttgccttg aataagaaaa tcttttacag tcttttagct taaaaaaaaa aaaaaaaaaa 960

aaaaaaaaa 969

<210> 2
<211> 251
<212> PRT
<213> DifE

<400> 2

Asn Ser Leu Asn Leu Asn Ile Thr Thr Lys Asp Phe Thr Glu Ala Met
1 5 10 15

Thr Gly Lys Thr Ala His Pro Gly Ile Leu Arg Ser Asp Ala Leu Arg
20 25 30

Lys Tyr Ile Leu Glu Thr Ser Val Tyr Pro Arg Glu His Glu Leu Leu
35 40 45

Lys Glu Leu Thr Lys Ala Ser Phe Glu Asn Tyr Lys Ala Ala Ser Phe
50 55 60

Met Gly Leu Pro Gln Asp Glu Ala Gln Phe Leu Ser Met Phe Leu Lys
65 70 75 80

Leu Ile Asn Ala Lys Lys Thr Leu Glu Ile Gly Val Phe Thr Gly Tyr
85 90 95

Ser Leu Leu Val Thr Ala Leu Ala Leu Pro Glu Asp Gly Lys Val Ile
100 105 110

Ala Ile Asp Pro Asp Arg Glu Ala Tyr Glu Val Gly Leu Pro Tyr Ile
115 120 125

Gln Lys Ala Gly Val Glu His Lys Ile Glu Phe Ile Gln Ser Glu Ala
130 135 140

Val Pro Val Leu Glu Lys Leu Leu Ser Asn Glu Lys Glu Ala Gly Thr
145 150 155 160

Phe Asp Phe Val Phe Ile Asp Ala Asp Lys Glu Asn Tyr Leu Lys Tyr
165 170 175

His Glu Ile Val Leu Lys Leu Val Lys Val Gly Gly Val Ile Gly Tyr
180 185 190

Asp Asn Thr Leu Trp Phe Gly Thr Val Ala Leu Ser Glu Asp Asp Pro
195 200 205

Met Pro Glu Gly Leu Arg Ala Leu Arg Gly His Val Met Lys Val Asn
210 215 220

Ser Phe Leu Ala Thr Asp Pro Arg Val Glu Val Ala Gln Leu Ser Ile
225 230 235 240

Gly Asp Gly Leu Thr Leu Cys Arg Arg Leu Ser
245 250

<210> 3
<211> 45
<212> DNA
<213> oligonucleotide

<400> 3
gagagagaga gagagagaga tctcgagttt ttttttttt ttttt 45

<210> 4
<211> 888
<212> DNA
<213> E20

<400> 4
cacttctcta ttttcttgaa aagcaccctc aatttgaata ttactaccaa ggattttact 60

gaagcaatga caggcaaaac cgcccaccct ggcattcttc gaagtgatgc cctcaggaag 120

tacattttgg aaacctcagt ttatccaaga gagcatgagc tactcaaaga actaacaaaa 180

gcttcattcg agaattataa agcagcgagc tttatgggtc ttcctcaaga tgaagcccag 240

tttctatcga tgttcctaaa gctcataaat gcaaagaaaa cactagagat tggagttttc 300

actggttact ctctgcttgt tactgctctt gctttgccag aagatgggaa agtaatagca 360

attgacccgg acagagaggc atatgaggtt ggattacctt atattcagaa ggctggtgtg 420

gaacataaga tcgagttcat tcaatcagaa gccgtgcccg ttcttgaaaa actcctctct 480

aacgagaaag aagcagggac atttgatttc gtgttcattg atgctgataa ggagaattat 540

ttgaagtacc atgagatagt gctaaaattg gtgaaagttg gaggagtgat aggatatgac 600

aacaccttat ggtttgggac agtggcactt tcagaggatg atccaatgcc agaaggttta 660

agagcattaa ggggacatgt tatgaaggtc aatagctttt tagctactga ccctcgtgtt 720

gaagtagctc aactttcaat tggtgatggc cttacccttt gcaggcgtct ctcctaggtc 780

cagttaattg gtgcaatgcc aagtcaacgc gaagatatgt actagatgta tgtcaggggt 840

tgaatttatt gaatttatgt tgttgagaag aaaaaaaaaa aaaaaaaa 888

<210> 5
<211> 263
<212> PRT
<213> E20

<400> 5

Ile Pro Leu Leu Ser His Phe Ser Ile Phe Leu Lys Ser Thr Leu Asn
1 5 10 15

Leu Asn Ile Thr Thr Lys Asp Phe Thr Glu Ala Met Thr Gly Lys Thr
20 25 30

Ala His Pro Gly Ile Leu Arg Ser Asp Ala Leu Arg Lys Tyr Ile Leu
35 40 45

Glu Thr Ser Val Tyr Pro Arg Glu His Glu Leu Leu Lys Glu Leu Thr
50 55 60

Lys Ala Ser Phe Glu Asn Tyr Lys Ala Ala Ser Phe Met Gly Leu Pro
65 70 75 80

Gln Asp Glu Ala Gln Phe Leu Ser Met Phe Leu Lys Leu Ile Asn Ala
85 90 95

Lys Lys Thr Leu Glu Ile Gly Val Phe Thr Gly Tyr Ser Leu Leu Val
100 105 110

Thr Ala Leu Ala Leu Pro Glu Asp Gly Lys Val Ile Ala Ile Asp Pro
115 120 125

Asp Arg Glu Ala Tyr Glu Val Gly Leu Pro Tyr Ile Gln Lys Ala Gly
130 135 140

Val Glu His Lys Ile Glu Phe Ile Gln Ser Glu Ala Val Pro Val Leu
145 150 155 160

Glu Lys Leu Leu Ser Asn Glu Lys Glu Ala Gly Thr Phe Asp Phe Val

165 170 175

Phe Ile Asp Ala Asp Lys Glu Asn Tyr Leu Lys Tyr His Glu Ile Val
180 185 190

Leu Lys Leu Val Lys Val Gly Gly Val Ile Gly Tyr Asp Asn Thr Leu
195 200 205

Trp Phe Gly Thr Val Ala Leu Ser Glu Asp Asp Pro Met Pro Glu Gly
210 215 220

Leu Arg Ala Leu Arg Gly His Val Met Lys Val Asn Ser Phe Leu Ala
225 230 235 240

Thr Asp Pro Arg Val Glu Val Ala Gln Leu Ser Ile Gly Asp Gly Leu
245 250 255

Thr Leu Cys Arg Arg Leu Ser
260

<210> 6
<211> 1077
<212> DNA
<213> E33

<400> 6
attttctgga caagttcgat tacctttgaa tctccaagga ctttactgaa tcaatggcag 60

gcaaaagcgg acatggctcc attcttcaaa gtgaagccct caagaagtac atcttcgaaa 120

ctagtgtgta tccaagagaa cacgagcaac tcaaagaact cacacaagcc tcattcgata 180

agtataaaat agtgagcttg atgggtgtgc ctccagatga agcccaattt ctctcgatgc 240

tcttaaaaat aatgaatgca aagaagacaa tggagattgg agtttttacc ggttattctc 300

ttctggctac tgctcttgca ttgccagaag atggaaaaat tatagcgatt gatccggaca 360

gagaagcata tgaggttgga ttgccatata ttcagaaggc tggtgtggag cataagattg 420

aatttattca atcagaagcc ttaccagtac tcgaaaaact cctctaacgg tgaggaagaa 480

ggaacatttg atttcatatt cattgatgct gataaggaga actatctgaa gtaccatgag 540

atagtactaa aattggtgaa agtgggagga gtgataggct atgacaacac attatggttt 600

gggaccgtgg cactttcaga tgatgatcct ataccacaag gcttaagaga attgaggaga 660

tcggttttga agatcaacag ttttttagct actgatcctc gcattgaatt agctcatctt 720

tcaattggtg atggtcttac ccttggcagg cgtctcagct agtttatttt tcgtataatc 780

atctgaattc cggaatccat tatctttata gttttttgtt tttcagtact agtgatattt 840

ttcagtcccc acttatggat aacactgggt aatgagtatt gttgcagaag tagtgacatt 900

tttaagtttg gttcgtccat ctgctaaaga agtcacgatt tcgtcttgta gacgagctat 960

agtatgcatt tgcattttgg ttaatttcgc atgtagtgtt gaaatgtgaa ttaccaaaag 1020

caaaagtaat aaaatgttta catttgttgt gttttaaaaa aaaaaaaaaa aaaaaaa 1077

<210> 7
<211> 244
<212> PRT
<213> E33

<400> 7

Ile Ser Lys Asp Phe Thr Glu Ser Met Ala Gly Lys Ser Gly His Gly
1 5 10 15

Ser Ile Leu Gln Ser Glu Ala Leu Lys Lys Tyr Ile Phe Glu Thr Ser
20 25 30

Val Tyr Pro Arg Glu His Glu Gln Leu Lys Glu Leu Thr Gln Ala Ser
35 40 45

Phe Asp Lys Tyr Lys Ile Val Ser Leu Met Gly Val Pro Pro Asp Glu
50 55 60

Ala Gln Phe Leu Ser Met Leu Leu Lys Ile Met Asn Ala Lys Lys Thr
65 70 75 80

Met Glu Ile Gly Val Phe Thr Gly Tyr Ser Leu Leu Ala Thr Ala Leu
85 90 95

Ala Leu Pro Glu Asp Gly Lys Ile Ile Ala Ile Asp Pro Asp Arg Glu
100 105 110

Ala Tyr Glu Val Gly Leu Pro Tyr Ile Gln Lys Ala Gly Val Glu His
115 120 125

Lys Ile Glu Phe Ile Gln Ser Glu Ala Leu Pro Val Leu Glu Lys Leu
130 135 140

Leu Ser Asn Gly Glu Glu Glu Gly Thr Phe Asp Phe Ile Phe Ile Asp

145 150 155 160

Ala Asp Lys Glu Asn Tyr Leu Lys Tyr His Glu Ile Val Leu Lys Leu
165 170 175

Val Lys Val Gly Gly Val Ile Gly Tyr Asp Asn Thr Leu Trp Phe Gly
180 185 190

Thr Val Ala Leu Ser Asp Asp Asp Pro Ile Pro Gln Gly Leu Arg Glu
195 200 205

Leu Arg Arg Ser Val Leu Lys Ile Asn Ser Phe Leu Ala Thr Asp Pro
210 215 220

Arg Ile Glu Leu Ala His Leu Ser Ile Gly Asp Gly Leu Thr Leu Gly
225 230 235 240

Arg Arg Leu Ser

<210> 8
<211> 20
<212> DNA
<213> oligonucleotide

<400> 8
cttgctttgc cagaagatgg 20

<210> 9
<211> 32
<212> DNA
<213> oligonucleotide

<400> 9
gcatggatcc acaggcaaaa ccgcccaccc tg 32

<210> 10
<211> 31
<212> DNA
<213> oligonucleotide

<400> 10
gcatctgcag ctaggagaga cgcctgcaaa g 31

<210> 11
<211> 1006
<212> DNA

<213> TMT5.

<400> 11
ttcaattccg ccattttctc caataataac attcataaat acaatcagca gcagcaaaaa 60

tgaaagataa gttctatggc accattttgc agagcgaagc cctcgcaaag tatctgttag 120

agacaagtgc ctatccacga gaacatccgc agctcaaaga actaaggagc gcaactgtgg 180

acaagtatca atattggagc ttgatgaatg ttccagctga tgaggggcag ttcatttcaa 240

tgttactgaa aattatgaac gcaaaaaaga caattgaagt tggagttttc acaggctact 300

cactcctatc aactgctctg gctctacctg atgatggcaa aatcgttgcc attgatcctg 360

atagagaagc ttatgagact ggtttgccat ttatcaagaa agcaaacgtg gctcataaaa 420

tccaatacat acaatctgat gccatgaaag tcatgaatga cctcattgct gccaagggag 480

aagaagaaga ggggagcttt gactttgggt tcgtggatgc agacaaagaa aactacataa 540

actaccacga gaaactgttg aagctggtta aggttggagg gatcatagga tacgacaaca 600

ctctgtggtc tggaacagtt gctgcatctg aagacgatga gaataatatg cgagactact 660

taagaggttg cagagggcat atcctcaaac taaactcctt tctcgcaaac gatgatcgga 720

ttgaattggc tcacctctct attggagatg gactcacctt gtgcaaacgt ctcaaataat 780

aattttcaac tttattatta ttgtttcata aaaagcattt actgctggcc tggcctggcc 840

tgtttcagca tcttatattt ctattgttct aaatatttta gttatcttgt ttatcaactt 900

gtctgtctta tatgtttaaa agaaagatgt catgtaattg taactcgatc gggctcttgt 960

aatattataa tgaattttat tgattttcaa aaaaaaaaaa aaaaaa 1006

<210> 12
<211> 239
<212> PRT
<213> TFMT5

<400> 12

Met Lys Asp Lys Phe Tyr Gly Thr Ile Leu Gln Ser Glu Ala Leu Ala
1 5 10 15

Lys Tyr Leu Leu Glu Thr Ser Ala Tyr Pro Arg Glu His Pro Gln Leu
20 25 30

Lys Glu Leu Arg Ser Ala Thr Val Asp Lys Tyr Gln Tyr Trp Ser Leu
35 40 45

Met Asn Val Pro Ala Asp Glu Gly Gln Phe Ile Ser Met Leu Leu Lys
50 55 60

Ile Met Asn Ala Lys Lys Thr Ile Glu Val Gly Val Phe Thr Gly Tyr
65 70 75 80

Ser Leu Leu Ser Thr Ala Leu Ala Leu Pro Asp Asp Gly Lys Ile Val
85 90 95

Ala Ile Asp Pro Asp Arg Glu Ala Tyr Glu Thr Gly Leu Pro Phe Ile
100 105 110

Lys Lys Ala Asn Val Ala His Lys Ile Gln Tyr Ile Gln Ser Asp Ala
115 120 125

Met Lys Val Met Asn Asp Leu Ile Ala Ala Lys Gly Glu Glu Glu Glu
130 135 140

Gly Ser Phe Asp Phe Gly Phe Val Asp Ala Asp Lys Glu Asn Tyr Ile
145 150 155 160

Asn Tyr His Glu Lys Leu Leu Lys Leu Val Lys Val Gly Gly Ile Ile
165 170 175

Gly Tyr Asp Asn Thr Leu Trp Ser Gly Thr Val Ala Ala Ser Glu Asp
180 185 190

Asp Glu Asn Asn Met Arg Asp Tyr Leu Arg Gly Cys Arg Gly His Ile
195 200 205

Leu Lys Leu Asn Ser Phe Leu Ala Asn Asp Asp Arg Ile Glu Leu Ala
210 215 220

His Leu Ser Ile Gly Asp Gly Leu Thr Leu Cys Lys Arg Leu Lys
225 230 235

<210> 13
<211> 37
<212> DNA
<213> TMT5.BamHI.F

<400> 13
gcatggatcc aaagataagt tctatggcac cattttg 37

<210> 14
<211> 35
<212> DNA
<213> TMT5.PstI.R

<400> 14
gcatctgcag ttatttgaga cgtttgcaca aggtg 35

<210> 15
<211> 26
<212> DNA
<213> OMTIf2

<220>
<221> misc_feature
<222> (18)..(18)
<223> N = A or G or C or T

<220>
<221> misc_feature
<222> (24)..(24)
<223> N = A or G or C or T

<400> 15
accatcgaga tcggcgtntt ycangg 26

<210> 16
<211> 27
<212> DNA
<213> OMTIf4

<220>
<221> misc_feature
<222> (19)..(19)
<223> Y = C or T

<220>
<221> misc_feature
<222> (22)..(22)
<223> N = A or G or C or T

<220>
<221> misc_feature
<222> (25)..(25)
<223> Y = C or T

<400> 16
cgacttcgcc ttcgtggayg cngayaa 27

<210> 17
<211> 31
<212> DNA
<213> OMTIr3

<220>
<221> misc_feature
<222> (23)..(23)
<223> N = A or G or C or T

<220>
<221> misc_feature
<222> (26)..(26)
<223> N = A or G or C or T

<220>
<221> misc_feature
<222> (29)..(29)
<223> Y = C or T

<400> 17
tgaagttgat cttgtgctcc acnccngcyt t 31

<210> 18
<211> 26
<212> DNA
<213> OMTIr5

<220>
<221> misc_feature
<222> (17)..(18)
<223> N = A or G or C or T

<220>
<221> misc_feature
<222> (21)..(21)
<223> R = A or G

<220>
<221> misc_feature
<222> (24)..(24)
<223> N = A o rG or C or T

<400> 18
cgccggcaga aggtganncc rtcncc 26

<210> 19
<211> 45
<212> DNA

<213> dT(17)Ad2Ad1

<400> 19
ctgagagaac tagtctcgag ctctagaaca agctttttt ttttt 45

<210> 20
<211> 36
<212> DNA
<213> GI anchor

<220>
<221> misc_feature
<222> (24)..(25)
<223> I = deoxyinosine

<220>
<221> misc_feature
<222> (29)..(30)
<223> I = deoxyinosine

<220>
<221> misc_feature
<222> (34)..(35)
<223> I = deoxyinosine

<400> 20
ggccacgcgt cgactagtac gggnngggnn gggnng 36

<210> 21
<211> 780
<212> DNA
<213> Fuchsia FMT

<400> 21
accatcgaga tcggcgtctt cactggctat tctcttcttt gcactgcact tgctttgcct 60

cccgatggca agataacagc gatcgacccc gacaaagaag cttacgagac cgggctgcca 120

tttattcaga aagctggagt gggacataag atcaacttca tcaatggtga cgcacttgca 180

gtactcgacg atcttattgc agacggaaaa gatcaagagg ggagttttga ttttgcgttc 240

gtggatgcta acaaggaaga ttacatcaag taccacgaac agctgcttaa acttgtcaag 300

gtaggtggct tgatctgcta cgacaacacc ctgtggttcg ggtcggtggc gctctccgaa 360

gaagatccca tggacgagtt tatgagaagc ggcagggtcc cgcttaggaa gttgaacgac 420

ttcctcgcaa atgacccccg tatcgagtca tgccttgttt ccatcggtga tggcctcacc 480

ctctgccgcc gccgcctcta atgcatctcg agagagttac tggcccctag ctagctagct 540

agctcgttgt tgttatatat atattatccg attgatatgt ggattctcac catatgtacg 600

tggattctgc gtactatcca gtggcgcctt ttgttgcatc tatctatatt tctagtttat 660

tatatgtacc atattcgctt ccgatatgtg cgaataagtc ggatgccatg cttccgatgg 720

ggtatcgttc ttattttcaa tcgttagtga tttttaagtt gagcaaaaaa aaaaaaaaaa 780

<210> 22
<211> 166
<212> PRT
<213> Fuchsia FMT

<400> 22

Thr Ile Glu Ile Gly Val Phe Thr Gly Tyr Ser Leu Leu Cys Thr Ala
1 5 10 15

Leu Ala Leu Pro Pro Asp Gly Lys Ile Thr Ala Ile Asp Pro Asp Lys
20 25 30

Glu Ala Tyr Glu Thr Gly Leu Pro Phe Ile Gln Lys Ala Gly Val Gly
35 40 45

His Lys Ile Asn Phe Ile Asn Gly Asp Ala Leu Ala Val Leu Asp Asp
50 55 60

Leu Ile Ala Asp Gly Lys Asp Gln Glu Gly Ser Phe Asp Phe Ala Phe
65 70 75 80

Val Asp Ala Asn Lys Glu Asp Tyr Ile Lys Tyr His Glu Gln Leu Leu
85 90 95

Lys Leu Val Lys Val Gly Gly Leu Ile Cys Tyr Asp Asn Thr Leu Trp
100 105 110

Phe Gly Ser Val Ala Leu Ser Glu Glu Asp Pro Met Asp Glu Phe Met
115 120 125

Arg Ser Gly Arg Val Pro Leu Arg Lys Leu Asn Asp Phe Leu Ala Asn
130 135 140

Asp Pro Arg Ile Glu Ser Cys Leu Val Ser Ile Gly Asp Gly Leu Thr
145 150 155 160

Leu Cys Arg Arg Arg Leu

165

<210> 23
<211> 27
<212> DNA
<213> OMTIf1

<220>
<221> misc_feature
<222> (17)..(17)
<223> Y = C or T

<220>
<221> misc_feature
<222> (19)..(19)
<223> N = A or G or C or T

<220>
<221> misc_feature
<222> (22)..(22)
<223> R = A or G

<220>
<221> misc_feature
<222> (25)..(25)
<223> R = A or G

<220>
<221> misc_feature
<222> (26)..(26)
<223> Y = C or T

<400> 23
ccgggagcac gagcacytna argaryt 27

<210> 24
<211> 28
<212> DNA
<213> OMTIf3

<220>
<221> misc_feature
<222> (18)..(18)
<223> R = A or G

<220>
<221> misc_feature
<222> (21)..(21)
<223> R = A or G

<220>
<221> misc_feature
<222> (24)..(24)
<223> N = A or G or C or T

<220>
<221> misc_feature
<222> (27)..(27)
<223> N = A or G or C or T

<400> 24
ggcctgccct tcatccaraa rgcnggng 28

<210> 25
<211> 34
<212> DNA
<213> OMTIr4

<220>
<221> misc_feature
<222> (26)..(26)
<223> R = A or G

<220>
<221> misc_feature
<222> (32)..(32)
<223> R = A or G

<220>
<221> misc_feature
<222> (29)..(29)
<223> N = A or G or C or T

<400> 25
cgtggtagtt cacgtagttg ctcttrtcng crtc 34

<210> 26
<211> 1079
<212> DNA
<213> E.33

<400> 26
attttctgga caagttcgat tacctttgaa tctccaagga ctttactgaa tcaatggcag 60

gcaaaagcgg acatggctcc attcttcaaa gtgaagccct caagaagtac atcttcgaaa 120

ctagtgtgta tccaagagaa cacgagcaac tcaaagaact cacacaagcc tcattcgata 180

agtataaaat agtgagcttg atgggtgtgc ctccagatga agcccaattt ctctcgatgc 240

tcttaaaaat aatgaatgca aagaagacaa tggagattgg agtttttacc ggttattctc 300

ttctggctac tgctcttgca ttgccagaag atggaaaaat tatagcgatt gatccggaca 360

gagaagcata tgaggttgga ttgccatata ttcagaaggc tggtgtggag cataagattg 420

aatttattca atcagaagcc ttaccagtac tcgaaaaact cctctctaac ggtgaggaag 480

aaggaacatt tgatttcata ttcattgatg ctgataagga gaactatctg aagtaccatg 540

agatagtact aaaattggtg aaagtgggag gagtgatagg ctatgacaac acattatggt 600

ttgggaccgt ggcactttca gatgatgatc ctataccaca aggcttaaga gaattgagga 660

gatcggtttt gaagatcaac agtttttag ctactgatcc tcgcattgaa ttagctcatc 720

tttcaattgg tgatggtctt acccttggca ggcgtctcag ctagtttatt tttcgtataa 780

tcatctgaat tccggaatcc attatcttta tagtttttg tttttcagta ctagtgatat 840

ttttcagtcc ccacttatgg ataacactgg gtaatgagta ttgttgcaga agtagtgaca 900

tttttaagtt tggttcgtcc atctgctaaa gaagtcacga tttcgtcttg tagacgagct 960

atagtatgca tttgcatttt ggttaatttc gcatgtagtg ttgaaatgtg aattaccaaa 1020

agcaaaagta ataaaatgtt tacatttgtt gtgttttaaa aaaaaaaaaa aaaaaaaaa 1079

<210> 27
<211> 20
<212> DNA
<213> Ad1

<400> 27
ctgagagaac tagtctcgag 20

<210> 28
<211> 28
<212> DNA
<213> petD8#1

<400> 28
ccctcgagtt tctattttgt gtgtgttg 28

<210> 29
<211> 26
<212> DNA
<213> petD8#2

<400> 29
gggaattcta gagctcgagg atcacg 26

<210> 30
<211> 24
<212> DNA
<213> PMT-F

<400> 30
actaccaagg atcctactga agca 24

<210> 31
<211> 20
<212> DNA
<213> PMT-R

<400> 31
ctcgaatgaa gcttttgtta 20

<210> 32
<211> 24
<212> DNA
<213> TMT-F

<400> 32
cataaatagg atccgcagca gcaa 24

<210> 33
<211> 20
<212> DNA
<213> TMT-R

<400> 33
agtctcataa gcttctctat 20

<210> 34
<211> 22
<212> DNA
<213> FucR1

<400> 34
gcaagtgcag tgcaaagaag ag 22

<210> 35
<211> 20
<212> DNA
<213> FucR3

<400> 35
gatcttatgt tccactccgc 20

<210> 36
<211> 20

<212> DNA
<213> FucR5

<400> 36
gagagatctg accagtaagg 20

<210> 37
<211> 24
<212> DNA
<213> FucR6

<400> 37
ggatattttt cggccgtgac ctcc 24

<210> 38
<211> 24
<212> DNA
<213> FucF1

<400> 38
atcttagaga cgactgctta tccc 24

<210> 39
<211> 68
<212> DNA
<213> Tor-5' pos

<400> 39
aattcgcagc aaaaatgaaa gataagttct atggcaccat tttgcagagc gaagccctcg 60

caaagtat 68

<210> 40
<211> 63
<212> DNA
<213> Tor-5' neg

<400> 40
tactttgcga gggcttcgct ctgcaaaatg gtgccataga acttatcttt catttttgct 60

gcg 63

<210> 41
<211> 841
<212> DNA
<213> Fuchsia FMT (3282)

<400> 41
atcttagaga cgactgctta tcccggagaa aatgagcatc tgaagcaact ccgggaggtc 60

acggccgaaa aatatcctta ctggagcatg atgaatgtgt caattgacga gggacaactt 120

atatcgctaa tattgaagct catgaacgcg agaaagacat tagagatcgg cgtcttcact 180

ggctattctc ttctttgcac tgcacttgct ttgcctcccg atggcaagat aacagcgatc 240

gaccccgaca aagaagctta cgagaccggg ctgccattta ttcagaaagc tggagtggaa 300

cataagatca acttcatcaa tggtgacgca cttgcagtac tcgacgatct tattgcagac 360

ggaaaagatc aagaggggag ttttgatttt gcgttcgtgg atgctaacaa ggaagattac 420

atcaagtacc acgaacagct gcttaaactg gtcaaggtag gtggcttgat ctgctacgac 480

aacaccctgt ggttcgggtc ggtggcgctc tccgaagaag atcccatgga tgagtttatg 540

aggagcggca gggtcccaat taggaagttg aacgacttcc tcgcaaatga cccccgtatc 600

gagtcatgcc ttgtttccat cggtgatggc atcaccctct gccgccgccg cctctaatgc 660

atctcgagag agttactggc ccctagctag ctagctcgtt gttgttatat atatatatta 720

tccgattgat atgtggattc tcaccatatg tacgtggatt ctgtgtacta tccagtggcg 780

ccttttgttg catctatcta tatttctagt ttattttatg taccaaaaaa aaaaaaaaaa 840

a 841

<210> 42
<211> 218
<212> PRT
<213> Fuchsia FMT (3282)

<400> 42

Ile Leu Glu Thr Thr Ala Tyr Pro Gly Glu Asn Glu His Leu Lys Gln
1 5 10 15

Leu Arg Glu Val Thr Ala Glu Lys Tyr Pro Tyr Trp Ser Met Met Asn
20 25 30

Val Ser Ile Asp Glu Gly Gln Leu Ile Ser Leu Ile Leu Lys Leu Met
35 40 45

Asn Ala Arg Lys Thr Leu Glu Ile Gly Val Phe Thr Gly Tyr Ser Leu
50 55 60

Leu Cys Thr Ala Leu Ala Leu Pro Pro Asp Gly Lys Ile Thr Ala Ile
65 70 75 80

Asp Pro Asp Lys Glu Ala Tyr Glu Thr Gly Leu Pro Phe Ile Gln Lys
85 90 95

Ala Gly Val Glu His Lys Ile Asn Phe Ile Asn Gly Asp Ala Leu Ala
100 105 110

Val Leu Asp Asp Leu Ile Ala Asp Gly Lys Asp Gln Glu Gly Ser Phe
115 120 125

Asp Phe Ala Phe Val Asp Ala Asn Lys Glu Asp Tyr Ile Lys Tyr His
130 135 140

Glu Gln Leu Leu Lys Leu Val Lys Val Gly Gly Leu Ile Cys Tyr Asp
145 150 155 160

Asn Thr Leu Trp Phe Gly Ser Val Ala Leu Ser Glu Glu Asp Pro Met
165 170 175

Asp Glu Phe Met Arg Ser Gly Arg Val Pro Ile Arg Lys Leu Asn Asp
180 185 190

Phe Leu Ala Asn Asp Pro Arg Ile Glu Ser Cys Leu Val Ser Ile Gly
195 200 205

Asp Gly Ile Thr Leu Cys Arg Arg Arg Leu
210 215

<210> 43
<211> 943
<212> DNA
<213> Fuchsia FMT full (3289)

<400> 43
aattcgcagc aaaaatgaaa gataagttct atggcaccat tttgcagagc gaagccctcg 60

caaagtatat cttagagacg accgcttatc ccggagaaaa tgagcatctg aagcaactcc 120

gggaggtcac ggccgaaaaa tatccttact ggagcatgat gaatgtgtca attgacgagg 180

gacaacttat atcgctaata ttgaagctca tgaacgcgag aaagacatta gagatcggcg 240

tcttcactgg ctattctctt ctttgcactg cacttgcttt gcctcccgat ggcaagataa 300

cagcgatcga ccccgacaaa gaagcttacg agaccgggct gccatttatt cagaaagctg 360

gagtggaaca taagatcaac ttcatcaatg gtgacgcact tgcagtactc gacgatctta 420

ttgcagacgg aaaagatcaa gaggggagtt ttgattttgc gttcgtggat gctaacaagg 480

aagattacat caagtaccac gaacagctgc ttaaactggt caaggtaggt ggcttgatct 540

gctacgacaa caccctgtgg ttcgggtcgg tggcgctctc cgaagaagat cccatggatg 600

agtttatgag gagcggcagg gtcccaatta ggaagttgaa cgacttcctc gcaaatgacc 660

cccgtatcga gtcatgcctt gtttccatcg gtgatggcat caccctctgc cgccgccgcc 720

tctaatgcat ctcgagagag ttactggccc ctagctagct agctcgttgt tgttatatat 780

atatattatc cgattgatat gtggattctc accatatgta cgtggattct gtgtactatc 840

cagtggcgcc ttttgttgca tctatctata tttctagttt attttatgta ccaaaaaaaa 900

aaaaaaaaag cttgttctac agctcgagac tagttctctc aaa 943

<210> 44
<211> 236
<212> PRT
<213> Fuchsia FMT full (3289)

<400> 44

Met Lys Asp Lys Phe Tyr Gly Thr Ile Leu Gln Ser Glu Ala Leu Ala
1 5 10 15

Lys Tyr Ile Leu Glu Thr Thr Ala Tyr Pro Gly Glu Asn Glu His Leu
20 25 30

Lys Gln Leu Arg Glu Val Thr Ala Glu Lys Tyr Pro Tyr Trp Ser Met
35 40 45

Met Asn Val Ser Ile Asp Glu Gly Gln Leu Ile Ser Leu Ile Leu Lys
50 55 60

Leu Met Asn Ala Arg Lys Thr Leu Glu Ile Gly Val Phe Thr Gly Tyr
65 70 75 80

Ser Leu Leu Cys Thr Ala Leu Ala Leu Pro Pro Asp Gly Lys Ile Thr
85 90 95

Ala Ile Asp Pro Asp Lys Glu Ala Tyr Glu Thr Gly Leu Pro Phe Ile
100 105 110

Gln Lys Ala Gly Val Glu His Lys Ile Asn Phe Ile Asn Gly Asp Ala
115 120 125

Leu Ala Val Leu Asp Asp Leu Ile Ala Asp Gly Lys Asp Gln Glu Gly
130 135 140

Ser Phe Asp Phe Ala Phe Val Asp Ala Asn Lys Glu Asp Tyr Ile Lys
145 150 155 160

Tyr His Glu Gln Leu Leu Lys Leu Val Lys Val Gly Gly Leu Ile Cys
165 170 175

Tyr Asp Asn Thr Leu Trp Phe Gly Ser Val Ala Leu Ser Glu Glu Asp
180 185 190

Pro Met Asp Glu Phe Met Arg Ser Gly Arg Val Pro Ile Arg Lys Leu
195 200 205

Asn Asp Phe Leu Ala Asn Asp Pro Arg Ile Glu Ser Cys Leu Val Ser
210 215 220

Ile Gly Asp Gly Ile Thr Leu Cys Arg Arg Arg Leu
225 230 235

<210> 45
<211> 8
<212> DNA
<213> oligonucleotide

<400> 45
gggatccc 8

<210> 46
<211> 8
<212> DNA
<213> oligonucleotide

<400> 46
ggcgcgcc 8

<210> 47
<211> 8
<212> DNA
<213> oligonucleotide

<400> 47
ggtcgacc 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/00079

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 15/29 C12N 15/11 A01H 5/00 C07H 21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE ELECTRONIC DATABASES

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE ELECTRONIC DATABASES

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DGENE, GenBank, GenPept (Seq Nos: 2, 5, 6, 7, 12, 22, 42, 44),

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GenPept Accession No: AAG52015 (15 June 2001) Putative S-adenosyl-L-methionine: trans-caffeoyl-Coenzyme A 3-O-methyltransferase. Lin, X. *et al.* (GAP: 60% identical with Seq ID 2, 5; 61% identical with Seq ID 7, 12, 42; 65% identical with Seq ID 22; 59% identical with Seq ID 44). See whole document.	1, 9-11, 13-20, 22-26, 61-67
X	Swiss-Prot Accession No: P93711 (15 July 1998) Caffeoyl-CoA O-methyltransferase. Kawai, S. *et al.* (GAP: 62% identical with Seq ID 2; 62% identical with Seq ID 5; 61% identical with Seq ID 7; 61% identical with Seq ID 12; 60% identical with Seq ID 44) See whole document.	1, 9-11, 13, 14, 17-20, 22, 23, 26, 61-67

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
16 April 2003

Date of mailing of the international search report
28 APR 2003

Name and mailing address of the ISA/AU

AUSTRALIAN PATENT OFFICE
PO BOX 200, WODEN ACT 2606, AUSTRALIA
E-mail address: pct@ipaustralia.gov.au
Facsimile No. (02) 6285 3929

Authorized officer

PHILIPPA WYRDEMAN
Telephone No : (02) 6283 2554

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/00079

C (Continuation). **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GenPept Accession No: CAC40584 (31 May 2001) Unnamed protein product [*Zea mays*]. Helent Jarvis, T.G. (GAP: 63% identical with Seq ID 22) See whole document. And WO01134817 (PIONEER HI-BRED INTERNATIONAL) 17 May 2001. See Sequence 13.	1, 9, 15, 18, 24, 61-67
X	GenPept Accession No: AAC26191 (15 July 1998) Caffeoyl-CoA 3-O-methyltransferase. De Melis, L.E. *et al.* (GAP: 60.7% identical with Seq ID 42). See whole document.	1, 9, 16, 18, 25, 61-67
X	Jonsson, L.M.V. *et al.* 1982. Methylation of anthocyanins by cell-free extracts of flower buds of *Petunia Hybrida*. Phytochemistry. 21(10):2457-2459. See whole document.	1-26
X	Jonsson, L.M.V. *et al.* 1984. Properties and genetic control of four methyltransferases involved in methylation of anthocyanins in flowers of *Petunia hybrida*. Planta. 160:174-179. See whole document.	1-26
X	Jonosson, L.M.V. *et al.* 1983. Genetic control of anthocyanin-O-methyltransferase activity in flowers of *Petunia hybrida*. Theoretical and Applied Genetics. 66:349-355. See whole document.	1-26
X	Gauthier, A. *et al.* 1998. Characterisation of two cDNA clones which encode O-methyltransferases for the methylation of both flavonoid and phenylpropanoid compounds. Archives of Biochemistry and Biophysics. 351(2):243-249. See whole document.	1, 2
A	Ibrahim, R.K. *et al.* 1998. Plant O-methyltransferases: molecular analysis, common signature and classification. Plant molecular Biology. 36:1-10. See whole document.	1-69
A	Joshi, C.P. *et al.* 1998. Conserved sequence motifs in plant S-adenosyl-L-methionine-dependent methyltransferases. Plant Molecular Biology. 37:663-674. See whole document.	1-69

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU03/00079

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	200134817	AU	200114652	EP	1226260	HU	200203221

END OF ANNEX